Proceedings of the 11th International Students Conference "Modern Analytical Chemistry"

Prague, 22—23 September 2015

Edited by Karel Nesměrák

Charles University in Prague, Faculty of Science
Prague 2015

Proceedings of the
11th International Students Conference
"Modern Analytical Chemistry"

Proceedings of the 11th International Students Conference "Modern Analytical Chemistry"

Prague, 22—23 September 2015

Edited by Karel Nesměrák

Charles University in Prague, Faculty of Science
Prague 2015

CATALOGUING-IN-PUBLICATION – NATIONAL LIBRARY OF THE CZECH REPUBLIC
KATALOGIZACE V KNIZE – NÁRODNÍ KNIHOVNA ČR

Modern Analytical Chemistry (11. : 2015 : Praha, Česko)
Proceedings of the 11th International Students Conference "Modern Analytical Chemistry" : Prague, 22–23 September 2015 / edited by Karel Nesměrák. – 1st edition – Prague : Charles University in Prague, Faculty of Science, 2015. – 242 s.
ISBN 978-80-7444-036-6 (brožováno)

543
▪ analytical chemistry
▪ proceedings of conferences
▪ analytická chemie
▪ sborníky konferencí

543 – Analytical chemistry [10]
543 – Analytická chemie [10]

The electronic version of the Proceedings is available at the conference webpage:
http://www.natur.cuni.cz/isc-mac/

ISBN 978-80-7444-036-6

Preface

This volume of conference proceedings involves 41 contributions presented at the 11th year of the international conference "Modern Analytical Chemistry" organized by our Department of Analytical Chemistry, Faculty of Science, Charles University of Prague on 22–23 September 2015. The mission of the conference is to provide a forum for presentation of achievements in the field of analytical chemistry by PhD students from various countries. Starting 2004 our gathering offers annually the chance for improvement of the presentation skills, provides the floor for discussion and exchange of experiences and opinions, and moreover stimulates the enhancement of the knowledge of English language of the participants.

The contributions are assorted by the name of presenting author and, as the reader will see, cover all branches of analytical chemistry, from improvement of instrumentation to application on environmental and toxicological problems. The Proceedings assure us, that analytical chemistry – thanks to young analytical chemists – remains exciting and steadily developing science with new, unsuspected ways of its innovation and application.

We are very grateful to the Division of Analytical Chemistry of EuCheMS for its auspices of our conference this year. Also, all sponsors are cordially thanked, not only for their kind financial sponsorship, but also for their continuous support and cooperation in many of our other activities.

Prof. RNDr. Věra Pacáková, CSc. RNDr. Karel Nesměrák, Ph.D.

Prof. RNDr. Jiří Barek, CSc.

The 11th International Students Conference "Modern Analytical Chemistry" is organized in cooperation with DAC EuCheMS.

EuCheMS
European Chemical Sciences
Division of Analytical Chemistry

Sponsors

The organizers of 11th International Students Conference "Modern Analytical Chemistry" gratefully acknowledge the generous sponsorship of following companies:

http://www.alsglobal.cz/

http://www.thermofisher.cz/

http://www.hpst.cz/

http://www.lach-ner.com/

http://www.shimadzu.eu/analytics

http://www.quinta.cz/

Contents

Contributions

Development of different methods for drugs and psychoactive substances extraction from hair samples and their identification based on HPLC-ESI-QTOF analysis

Justyna Aszyk*, Agata Kot-Wasik

Department of Analytical Chemistry, Chemical Faculty, Gdańsk University of Technology, Narutowicza Str. 11/12, 80-233, Gdańsk, Poland ✉ *aszykjustyna@gmail.com*

Keywords
drugs
fragmentation pathways
metabolites
liquid chromatography
quadrupole time-of-flight mass spectrometry

Abstract
Over the past decade, the use of non-controlled designer drugs and drug of abuse has rapidly increased. Hair, as a human matrix, enables detection of drugs incorporated into its structure. Studies of presence and identification of drug metabolites in human hair samples has been performed using liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry (HPLC-ESI-QTOF-MS) with steady alternation of MS and MS/MS. The comparison of three analytical procedures (including decontamination and extraction steps) allowing qualitative screening of drugs present in hair samples were performed based on hair samples collected from 13 volunteers declaring exposition to several drugs. It was concluded, that all methods are suitable to extract the majority of the relevant substances from hair. In spite of this, ambiguity correlation between data known from volunteers questionnaires and data obtained from HPLC-ESI-QTOF-MS analyses has been observed and discussed.

1. Introduction

Hair, as a human matrix, exhibit a lot of highlights in drug of abuse analysis compared to other biological samples (blood or urine) [1]. First of all, sampling step for hair is non-invasive, simple, and painless for patient. Secondly, hair sample does not require specialist storage and transport conditions due to slow process of destruction of hair compare to another biological samples [2]. Besides, drugs can stay in this matrix for a long time (even months). However, hair samples have got some limitations for analysts, just to mention time-consuming analytical procedures and high correlation to melanin concentration dramatically affecting results.

Hair analysis consists of few principal steps: sampling, storage and transport, decontamination, extraction of features from biological matrix, instrumental analysis and finally data interpretation. The decontamination phase consists of

one or more washing of the sample in order to eliminate possible external contamination. The extraction of the analytes from the hair can be achieved by various methods, which differ according to the nature of the analytes themselves and the identification technique to be employed [3].

The aim of this study was to evaluate and compare of three extraction procedures and to present the potential of liquid chromatography and time-of-flight mass spectrometry for qualitative drug screening in hair samples collected from 13 volunteers. HPLC-QTOF-MS and MS/MS offers high mass resolution and mass accuracy [4, 5].

2. Experimental

2.1 Chemicals

Acetonitrile, methanol, ammonium formate (LCMS grade) were purchased from Sigma Aldrich. Formic acid was purchased from Merck. Acetone and hexane (analytical grade) were purchased from POCH (Gliwice, Poland). Nylon ProFill™ 25 mm bright blue (0.2 μm pore size) syringe filters Whatman Puradisc™ 13 mm PTFE (0.2 μm pore size) syringe filters were purchased from Sigma Aldrich. Ultrapure water was prepared using HLP5 system from Hydrolab (Wiślina, Poland).

2.2 Sample preparation

Hair samples were collected from the posterior vertex region of volunteers. Additionally, control hair samples were taken for analytical purposes from persons who do not declare drug intake. The hair samples were stored in low density polyethylene Zipper bags in 20–25 °C until analysis. The decontamination of the hair was performed as follows: strand of hair (300 mg) was placed into an glass tube and decontaminated by gentle shaking in an ultrasonic bath using:

a) 10 ml *n*-hexane for 1 min followed by 10 ml of acetone for 1 min,
b) 10 ml deionised water for 1 min and two times for 10 min with 10 ml acetone,
c) 10 ml acetone for 2 min followed by 10 ml deionised water and again with 10 ml acetone.

After hair drying on a filter paper (up to 10 minutes) samples were cut to 12 cm pieces and homogenized. Then 200 mg of hair sample was placed in glass tube. Subsequently, hair samples were prepared according to three extraction procedures summarized in Table 1. After extraction, followed by centrifugation at 6000 rpm, liquid phase was collected and the excess of solvent was evaporated at 40 °C under a gentle nitrogen stream. The residue was reconstituted in a appropriate solvent according to applied procedure. Reconstitution in mixture of water:methanol with 0.05% of formic acid (95:5, v/v) resulted in colloid formation, therefore reconstitution in methanol was applied. It is worth to

Table 1
Workflow of extraction procedures for identification of xenobiotics in hair sample.

Parameter	Procedure 1	Procedure 2	Procedure 3
Assisted extraction factor	ultrasound	shaking	ultrasound
Temperature [°C]	50	37	37
Time of extraction [h]	1h, 18 h incubation	18	8
Amount and type of extraction solvent	10 ml of MeOH	5 ml of MeOH/-ACN/2 mM NH_4HCOOH (25:25:50, v/v/v)	10 ml of MeOH/ACN (50:50, v/v)
Solvent for reconstitution of residue	H_2O/MeOH with 0.05% formic acid (95:5,v/v)	MeOH	H_2O/MeOH (50:50, v/v)
Reference	[2]	[4]	[6]

mention that the colloid formation was recorded after two days extract storage at room temperature. Finally, 5 µl of the extract was injected for HPLLC-ESI-QTOF--MS analysis.

2.3 Instrumentation

The HPLC-ESI-Q-TOF-MS analysis was performed on an Agilent 1290 LC system equipped with a binary pump, an online degasser, an autosampler and a thermostated column compartment coupled with a 6540 Q-TOF-MS with a Dual ESI source (Agilent Technologies). An Agilent ZORBAX XDB-C-8, 150 mm × 4.6 mm, 5 µm column was used for RP-HPLC separation. The mobile phase consisted of mixture of water containing 0.05% formic acid (component A) and acetonitrile, methanol or methanol:acetonitrile (50:50, v/v) (component B). The gradient elution program was from 10 to 100% B during 20 minutes followed by 100% B maintained for 10 min. The column temperature throughout the separation process was kept at 40 °C. The mobile phase flow rate was 0.5 ml/min and the injection volume was 5 µl. During the analysis, the samples were kept in an autosampler at 4 °C. The ESI source was operated both in positive and negative ion mode with the following conditions: the fragmentor voltage was set at 80 or 150 V, nebulizer gas was set at 35 psi, capillary voltage was set at 3500 V, and drying gas flow rate and temperature were set at 10 l/min and 300 °C, respectively. The data were acquired in centroid and profile mode using High Resolution mode (4 GHz). The mass range was set at 100–1000 *m/z* in MS and MS/MS mode. The TOF-MS was calibrated on a daily basis.

Workflow for identification of drugs in hair sample is shown in Figure 1.

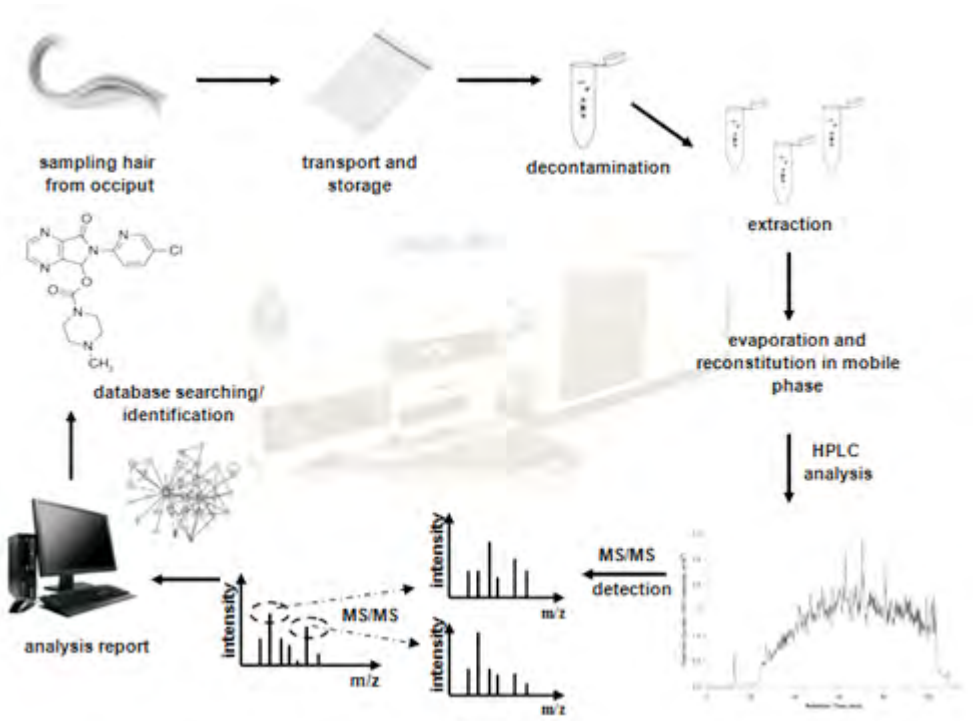


Fig. 1. Workflow for identification of xenobiotics in hair sample

3. Results and discussion

In the present study a comparison of the three developed analytical procedures was performed for 13 hair samples. The identification of drugs was based on QTOF-MS and QTOF-MS/MS data and comparison of theoretically calculated mass based on molecular formula with experimental mass obtained. High mass accuracy confirmed the trueness of obtained results. The results were summarized in Table 2.

It can be generally confirmed that three presented methods provided sufficient extraction recovery of the majority of drugs from hair samples allowing qualitative screening. Additionally, metabolites of tramadol (*O*-desmethyltramadol and *N*-desmethyltramadol) and metabolites of doxepin (hydroxydoxepin and desmethyldoxepin) were found in hair samples. According to similarity in doxepin structure (m/z = 280.1696) and desmethyldoxepin (m/z = 266.1539) these analytes were eluted at the same time. The intensity of hydroxydoxepin peak is higher than the intensity of doxepin peak, what can be a proof for higher incorporation of this metabolite in hair structure than other features. However, ultrasound assisted extraction exhibited some limitations. Long-lasting ultrasonication and temperature 50 °C resulted that perazine could not be detected probably due to the decomposition of the drug.

Table 2
Summarize of drug detected in hair samples and their theoretical and experimental masses.

Drug/metabolite	Teoretical mass [*m/z*]	Experimental mass [*m/z*]	Mass accuracy [ppm]
6-APB	176.1070	176.1069	0.57
6-APBD	178.1226	178.1230	2.25
amphetamine	136.1121	136.1124	2.20
dextromethorphan	272.1998	272.2009	4.04
dimethyltryptamine	189.1386	189.1382	2.11
doxepin	280.1696	280.1692	1.43
desmethyldoxepin	266.1539	266.1542	1.13
hydroxydoxepin	296.1645	296.1652	2.36
fentanyl	337.2274	337.2267	2.08
fluoxetine	310.1413	310.1415	0.64
hydroxyzine	375.1834	375.1830	1.07
methadone	310.2165	310.2170	1.61
methoxetamine	248.1645	248.1646	0.40
α-methyltryptamine	175.1230	175.1231	0.57
metropolol	268.1907	268.1899	2.98
noopept	319.1652	319.1656	1.25
paracetamol	152.0706	152.0704	1.32
perazine	340.1842	340.1833	2.65
sulfamethoxazole	254.0594	254.0590	1.57
α-tramadol	264.1958	264.1951	2.65
O-desmethylotramadol *N*-desmethylotramadol	250.1801	250.1799	0.80
trimethoprim	291.1452	291.1447	1.72
zopiclone	389.1123	389.1120	0.77
UR-144	312.2322	312.2310	3.84

However, there were 22 drugs or psychoactive substances, which were mentioned in volunteer's questionnaires, but were not detected in the hair samples. They were pointed in Table 3. Few reasons can be used to explain this phenomena just to mention washing-out by frequency use of shampooing and color of hair (sample III). It is well known, that the incorporation of drugs in the hair depends on melanin content in the matrix and is regulated by the pharmacological principles of the substance distribution. The incorporation and binding of drugs in the hair is much greater in pigmented versus non-pigmented hair, so no detection of these drugs in grey hair is explicable [2]. The reason can also lie in irregular intake of drugs (sample IX), insufficient stability of features in hair, a long-term medical treatment in case of some drugs and finally on low concentration of drug in hair sample not sufficient for QTOF-MS detection (sample VIII) [4]. In case of sample IX, hair were collected from tip (distal) section of hair as well. This additional analysis was performed in order to verify how cutting/not-cutting of hair for 5 years (as was declared in questionnaire) will affect results. This effort allowed to detect 6-APB (this drug was not detected in hair sample

Table 3
Summary of analysed hair samples with undetected substances.

Sample	Color of hair	Undetected substances	Note
III	grey	tolterodine	Washing hair at least one time per day
VIII	dark blonde	morphine	
IX	dark blonde	AM-2201, codeine, ephedrine, 4-AcO-DMT, methandienone, GBL, 2C-B, dimenhydrinate, 25-C-NBOMe, 4-FMA, 5-Meo-MiPT	The volunteer declared that since five years he did not cut hair.
XIII	middle blonde	ergine, 5-API, ethylphenidate, 2C-P, MAM-2201, 25C-NBOMe, MDMA, 25I-NBOMe, 5-HO-DMT	

taken from posterior vertex of the head), what confirms hypothesis that this drug was intaken in earlier period of life.

4. Conclusions

Broad spectrum toxicological screening of hair looks applicable to the search of many drugs of abuse. HPLCQTOF-MS can be successfully applied for the general unknown drug screening in hair. However, appropriate sample preparation method for drug isolation and concentration from hair samples must be used.

Acknowledgments

The authors would like to thank Patrycja Ratyńska for her great support; some of the results described herein were performed as part of her master thesis.

References

[1] Kłys M., Rojek S., Kulikowska J., Bożek E., Ścisłowski M.: *J. Chromatogr. B* **854** (2007), 299–307.
[2] Baciu T., Borrull F., Aguilar C., Calull M. :*Anal. Chim. Acta* **856** (2015), 1–26.
[3] Vogliardi S., Tucci M., Stocchero G., Ferrara S.D., Favretto D.: *Anal. Chim. Acta* **857** (2015), 1–27.
[4] Broecker S., Herre S., Pragst F. : *Forensic Sci. Int.* **218** (2012), 68–81.
[5] Bijlsma L., Sancho J.V., Hernández F., Niessen W.M.: *J. Mass Spectrom.* **46** (2011), 865–875.
[6] Domínguez-Romero J.C., García-Reyes J.F., Molina-Díaz A.: *J. Chromatogr. B* **879** (2011), 2034–2042.

Comparison of enzymatic and GC-MS/MS analysis of creatinine in urine samples

Viktória Ferenczy[a, b] *, Peter Kotora[a], Dušana Hudecová[a], Jaroslav Blaško[a], Róbert Kubinec[a], Darina Behúlová[b]

[a] *Comenius University, Faculty of Natural Sciences, Chemical Institute, Mlynská dolina, Ilkovičova 6, 842 15 Bratislava, Slovakia* ✉ *ferenczy@fns.uniba.sk*
[b] *University Children's Hospital, Department of Laboratory Medicine, Centre of Inherited Metabolic Diseases, Limbová 1, 833 40 Bratislava, Slovakia*

Keywords
creatinine
enzymatic analysis
GC-MS/MS
silylation
urine

Abstract
Work was focused on the comparison of enzymatic and gas chromatographic tandem mass spectrometric analysis of creatinine and the simultaneous analysis of creatinine and fructose with GC-MS/MS after silylation with hexamethyldisilazaneand *N,O*-bis(trimethylsilyl)trifluoroacetamide. This method allows the direct derivatization of urine samples without sample pretreatment before derivatization. Because of this, the analysis and the diagnostics of diseases are faster than in case of a separate determination of both analytes.

1. Introduction

Urine as biological fluid is a very good indicator of human health. Collection of urine is easier than collection of blood and avoids invasive procedures [1]. Urinary water-soluble organic compounds are the end products or intermediates of the metabolism of sugars, lipids, amino acids, etc. Creatinine is a cyclic nitrogen--containing organic substance formed by cyclization of creatine. Production and secretion of creatinine is normally in balance and any filtered creatinine by kidneys is eliminated from the body by urine [2]. Urinary creatinine excretion is a function of lean body mass in normal persons and shows little or no response to dietary changes. Creatinine in urine is used since several decades for the correction of excretion rates or urinary concentrations of numerous endogenous and exogenous substances [3].

Creatinine is a commonly analyzed compound in clinical biochemical laboratories. The most common and routine analytical method for the determination of creatinine is enzyme-catalyzed photometric assay.

Gas chromatography-mass spectrometry is frequently used for the measurement of many analytes [4], but the potential problem with GC-MS is the need of derivatization for polar and non-volatile analytes. Derivatization is based on the substitution of polar groups of carbohydrates in order to increase their volatility.

One of the most popular derivatization methods is silylation, when volatile and stable trimethylsilyl (TMS) ethers are formed [5]. Analysis of aqueous samples is problematic because of the high reactivity of silylation agents towards water [6].

Simultaneous determination of hydroxyl groups in fructose and amino groups in creatinine in aqueous samples was resolved with two step derivatization, first with hexamethyldisilazane (HMDS) and second with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). Comparison of enzymatic method was carried out with newly developed GC-MS/MS method.

2. Experimental

2.1 Reagents and chemicals

N,O-bis(trimethylsilyl)trifluoroacetamide, creatinine, and fructose were bought from Sigma-Aldrich. Trifluoroacetic acid was purchased from Fluorochem Ltd. (Hadfield, UK). Millipore HPLC water, acetonitrile, HMDS were bought from Merck. Methyl-4-deoxy-4-fluoro-α-D-glycopyranoside as an internal standard was purchased from SynthCluster s.r.o. (Modra, Slovakia).

2.2 Instrumentation

The GC-MS/MS analyses were carried out with a Trace GC Ultra gas chromatograph with a TriPlus autosampler and a TSQ Quantum XLS mass spectrometer (Thermo Fisher). The injected volume of sample was 1 μL into the injector operating in splitless mode (2 min). The injector temperature was 280 °C and the MS-transfer line was 260 °C. Compounds were separated on a 30 m × 0.25 mm (i.d.) × × 0.25 μm DB-5MS capillary column (Agilent Technologies). The column temperature was initially set to 80 °C, held for 1 min and increased at a rate of 20 °C min^{-1} to 210 °C and then increased to 230 °C at a rate of 2 °C min^{-1}.

The enzymatic analysis was carried out with a VITROS 5,1 FS Chemistry Systems (Ortho-Clinical Diagnostics) with VITROS CREA Slide method and the VITROS Chemistry Products Calibrator Kit 1 in the Department of Laboratory Medicine University Children's Hospital in Bratislava (Slovakia). The VITROS CREA Slide is a multilayered, analytical element coated on a polyester support. A 10 μL drop of urine sample is deposed on the slide and is distributed by the spreading layer and the slide is incubated at 37 °C. The resulting change in reflection density is measured at 2 time points. The analysis was 5 min. at 37 °C and the wavelength was 670 nm.

2.3 Derivatization

A two-step derivatization method was based on the previously developed method by Podolec et. al [6] with HMDS and BSTFA. In the first step, 700 μL of HMDS:ACN

mixture (1:1, v/v) was added as a silylation agent to 20 μL of urine sample for the derivatization of easily silylable functional groups (hydroxyl in fructose) and water, 2 μL of trifluoroacetic acid was added as a catalyst and the sample was heated to 50 °C for 30 min at 700 rpm in a Thermoshaker TS-100C from Biosan (Riga, Latvia) in open vial because of the escaping an ammonia gas produces in the reaction. In the second step, 300 μL of pure BSTFA was added and the mixture was heated to 80 °C for 30 min in a closed vial.

3. Results and discussion

The analyte concentration in urine was found by using internal standard calibration graph; the calibration curve was prepared in the concentration range 0.25–10,000 mg L^{-1}. The calibration curves were constructed based on the peak area ratio of the analytes and internal standard versus the concentration of the analytes in mg L^{-1}.

For the GC-MS/MS analysis, the derivatization method for the simultaneous analysis of carbohydrates and amines allows direct silylation of urine samples without sample pretreatment before derivatization (extraction, freeze-drying, etc.). A single run silylation of sugars and amino acids is problematic due to the different ability of the functional groups to form TMS derivatives. Powerful BSTFA enables the silylation of most functional groups, but its use leads to the formation of multiple derivatives of carbohydrates. Weaker HMDS resulted only in the expected derivatives of carbohydrates, but was inefficient in the derivatization of amino groups. By using a two-step silylation with HMDS and BSTFA, all the selected analytes were derivatized to their TMS analogs with only the expected derivatives formed.

The enzymatic analysis of creatinine which is used in University Children's Hospital is one of the most frequently used routine biochemical methods. Creatine is converted to sarcosine and urea by creatine amidinohydrolase. The sarcosine, in the presence of sarcosine oxidase, is oxidized to glycine, formaldehyde and hydrogen peroxide. The final reaction involves the peroxidase-catalyzed oxidation of a leuco dye to produce a colored product.

The comparison of enzymatic and GC-MS/MS analysis is shown in Fig. 1; the GC-MS/MS method for the determination of creatinine is comparable with the enzymatic method used in the hospital laboratory. In the laboratories of Chemical Institute we performed the simultaneous derivatization of creatinine and fructose. The GC-MS/MS chromatograms of real urine samples are shown in Fig. 2 and the analyte concentrations are listed in Table 1.

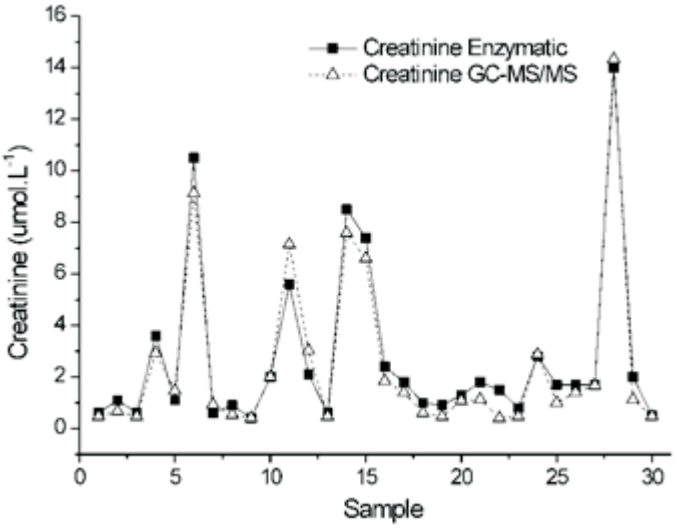


Fig. 1. Comparison of en-zymatic and GC-MS/MS analysis of creatinine in urine samples.

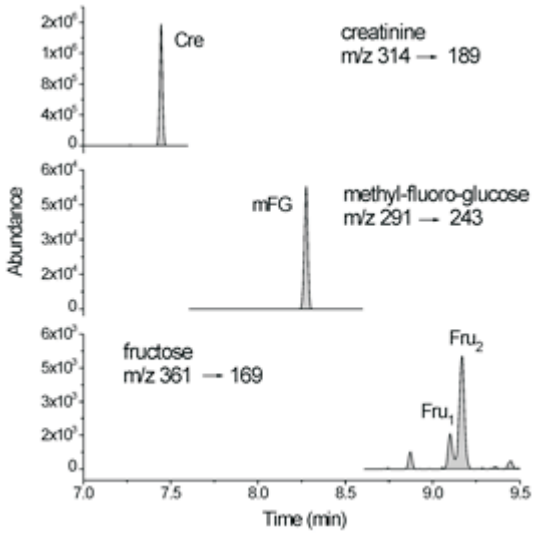


Fig. 2. GC-MS/MS chromatograms of TMS derivatives in urine samples: creatinine (Cre), methyl-4--deoxy-4-fluoro-α-D-glycopyranoside (mFG), β-D-fructofuranoside (Fru_1), α-D-fruc-tofuranoside (Fru_2).

Table 1
Concentrations of creatinine and fructose in various urine samples after GC-MS/MS analysis.

Analyte	Sample								Control samples			
	1		2		3		4					
	mg L^{-1}	µmol $mmol^{-1}$ of creatinine	mg L^{-1}	µmol $mmol^{-1}$ of creatinine	mg L^{-1}	µmol $mmol^{-1}$ of creatinine	mg L^{-1}	µmol $mmol^{-1}$ of creatinine	mg L^{-1}	RSD [%]	mg L^{-1}	RSD [%]
Creatinne	1550	13700	182	16100	183	16200	1560	13800	220	124	19500	124
Fructose	83.3	33.8	21.7	74.8	60.6	208	38.1	15.4	14.2	111	53.3	148

4. Conclusions

Comparison of these two methods shows, that the GC-MS/MS method with direct silylation enables simultaneous analysis of several analytes (creatinine and fructose) in one step, unlike the enzymatic analysis, when it is necessary to carry out a separation of all analytes separately in multiple steps.

Acknowledgments

This publication is the result of the project implementation: Comenius University in Bratislava Science Park supported by the Research and Development Operational Programme funded by the ERDF. Grant number: ITMS 26240220086.

References

[1] Matsumoto I., Kuhara T.: *Mass Spectrom. Rev.* **15** (1996), 43–57.

[2] Fraselle S., De Cremer K., W. Coucke W., Glorieux G., Vanmassenhove J., Schepers E., Neirynck N., Van Overmeire I., Van Loco J., Ban Biesen W., Vanholder R.: *J. Chromatogr. B* **988** (2015), 88–97.

[3] Tsikas D., Wolf A., Mitschke A., Gutzki F.M., Will W., Bader M.: *J. Chromatogr. B* **878** (2010), 2582–2592.

[4] Wajner M., de Moura D., Coelho R., Ingrassia R., Büker de Oliveira A., Brandt Busanello E.N., Raymond K., Pires R.F., Fischinger Moura de Souza C., Giuliani R., Regla Vargas C.: *Clin. Chim. Acta* **400** (2009), 77–81.

[5] Christou C., Gika H.G., Raikos N., Theodoridis G.: *J. Chromatogr. B* **964** (2014), 195–201.

[6] Podolec P., Hengerics Szabó A., Blaško J., Kubinec R., Górová R., Višňovský J., Gnipová A., Horváth A., Bierhanzl V., Hložek T., Čabala R.: *J. Chromatogr. B* **967** (2014), 134–138.

Cholesterol capillary monolithic columns for reversed-phase liquid chromatography

Damian Grzywiński*, Michał Szumski, Bogusław Buszewski

Department of Environmental Chemistry and Bioanalytics, Faculty of Chemistry, Nicolaus Copernicus University in Toruń, 7 Gagarina St, 87-100 Torun, Poland ✉ damciu@doktorant.umk.pl
Center for Modern Interdisciplinary Technologies, 4 Wilenska St, 87-100 Torun, Poland

Keywords
cholesterol stationary phase
micro-liquid chromatography
monoliths

Abstract
In this work were described the methodology of preparation of cholesterol monolithic capillary columns and their applications. This polymeric material was obtained in one-step thermally initiated polymerization carried out directly in the fused silica capillaries. As a functional monomer cholesteryl methacrylate was used and trimethylolpropane trimethacrylate was a cross-linker. As a porogenic solution isooctane/toluene mixture was used. The synthesized capillary columns were applied for the separation of alkylbenzenes, *o*-terphenyl/triphenylene, steroid hormones and polycyclic aromatic hydrocarbons.

1. Introduction

The monolithic columns (also called monoliths, continuous porous beds, polymer rods) have been existing in the literature since 1980s and early 1990s [1, 2]. There are generally distinguished two groups of monoliths: organic and inorganic materials [3]. The inorganic, silica-based monoliths can be obtained by immobilization of the silica particles by sintering or in the hydrolytic polycondensation of alkoxysilanes in the sol-gel process [4]. The organic monoliths are obtained in a single step (usually) polymerization of functional monomers and cross-linking reagents in the presence of a porogen solution and an initiator [3]. Initiation of polymerization can occur under the influence of heat, UV or γ radiation and chemical reaction [2]. Morphology of the obtained monolith depends on the method of initiation, temperature, the composition of the polymerization mixture and the type of porogen solution (molar or weight ratio of the functional monomer to cross-linking, monomer to porogen ratio, amount of initiator and so on) [5].

Monolithic columns can be an alternative for packed columns, but packed columns are still more popular as far. Actually, most of the chromatographic columns are packed with silica-based stationary phases. The silica gel can be modified with hydrophobic hydrocarbon chains of various lengths (C_2, C_8, C_{18}, C_{22}, C_{30}), polar groups (–NH_2, –CN, –DIOL) or non-polar moieties such as cholesterol [6]. Cholesterol stationary phases based on the silica-gel support have been used

in liquid chromatography since 1990s [7]. This stationary phase can be successfully used to separate, e.g. structural isomers [8], different xenobiotics [9], benzodiazepines [10], flavonoids [11], polycyclic aromatic hydrocarbons (PAHs) [6, 12], steroids[13], beta-blockers [14], tetracyclines [15], catechins, saikosaponins, carotens, vitamin K isomers [16, 17].

2. Experimental

2.1 Chemicals and reagents

The following chemicals were purchased from Sigma-Aldrich: trimethylolpropane trimethacrylate (TRIM), 2,2,4-trimethylpentane (isooctane), tetrahydrofuran, 3-(trimethoxysilyl)propyl methacrylate (γ-MAPS), thiourea, benzene, toluene, ethylbenzene, propylbenzene, butylbenzene, o-terphenyl, triphenylene, the steroid hormones standards and the PAHs standards. The radical polymerization initiator 2,2'-azoisobutyronitrile (AIBN) was from Fluka. Cholesteryl methacrylate (CholMA) we synthesized in our laboratory. Acetonitrile (HPLC ultra grade), methanol (HPLC ultra grade) were from J.T. Baker (Witko, Łódź, Poland). Deionized water was produced in our laboratory using a Milli-Q system (Millipore). Polyimide-coated fused silica capillaries of various diameters were purchased from Polymicro Technologies (USA).

2.2 Equipment

All chromatographic measurements were performed on a capillary LC system consisting of a pump delivering the mobile phase (Agilent 1260 cap pump with degasser, Agilent Technologies), 10-port valve with a microelectric actuator (C72MX-4694EH, Vici Valco Instruments) with a 50 nl external capillary loop, a set of connecting capillaries (TSP of various diameters Polymicro Technologies). On-column detection was performed using a Spectra-100 (Thermo Separations Products) detector. UV absorbance was monitored at 205 nm, 222 nm and 254 nm. For process control and data collection the Clarity 4.0.04.987 software was used (DataApex, Czech Republic).

The monolithic columns were characterized using scanning electron microscope (Leo 1430 VP, Leo Electron Microscope).

2.3 Synthesis of monolithic columns

Before polymerization, a polyimide-coated fused silica capillaries (180 μm I.D, 350 μm O.D.) were modified according to the general procedure reported earlier [18].

Monolithic stationary phases were prepared by one-step in-situ thermal polymerization carried out in a water bath at 60 °C for 24 h. The detailed

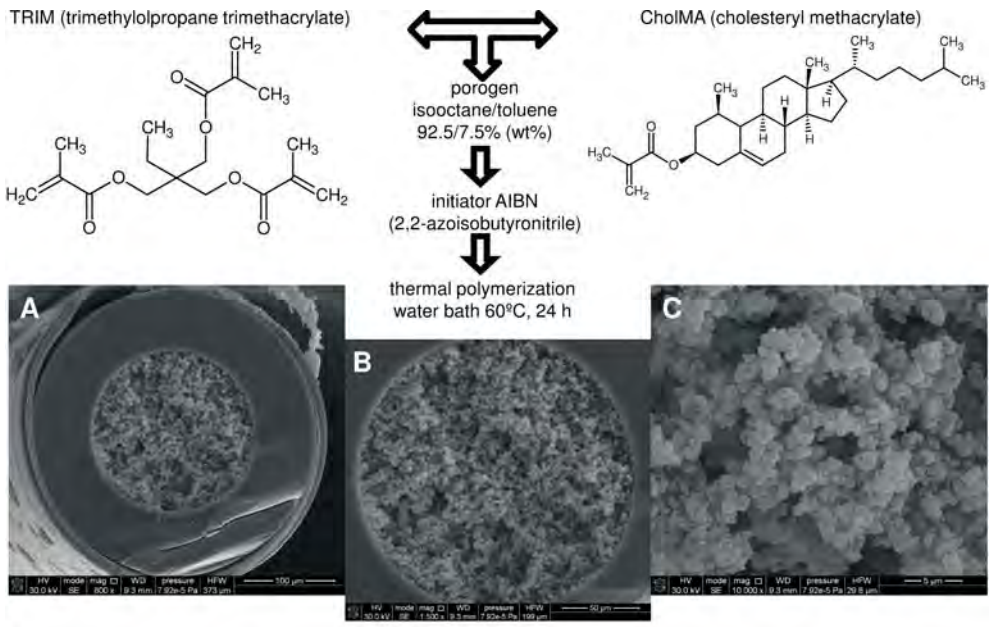


Fig. 1. Scheme of preparation of cholesterol monoliths and SEM micrographs of the obtained capillary monolithic columns at different magnification: (A) 800×, (B) 1,500×, (C) 10,000×. Compositions of monoliths: 12.5% CholMA, 27.5% TRIM, 55.5% isooctane, 4.5% toluene.

procedure of preparation of the cholesterol-based monolithic capillary columns has been reported previously [18]. Briefly, the following constituents ratios were used to synthesize the columns used in this work: TRIM/CholMA (27.5/12.5% by weight), porogen solvent: isooctane/toluene (92.5/7.5% by weight), and initiator AIBN (1% with respect to the monomers); see Fig. 1). The polymerization mixture was prepared in a 2 ml eppendorf vial, vortexed and degassed by sonification. Then, part of the reaction mixture was introduced into the capillary and after that the capillary was plugged with rubber septa at both ends and placed in a water bath. After the polymerization was completed, 5 mm was cut from each end of the capillaries, which were thereafter flushed with THF and ACN for at least 1 h at 200 bars of inlet pressure. The final column sizes were length 30.5 cm and d_c = 180 μm.

3. Results and discussion

3.1 Separations of small molecule compounds

Separation of an alkylbenzenes mixture was a basic assessment of the obtained columns and served to compare them to other cholesterol stationary phases (capillary columns packed with amino-cholesterol and diamino-chlesterol silica based stationary phases) as well as octadecyl methacrylate capillary monolithic column. In Fig. 2 we can see, that the separations of alkylbenzenes using

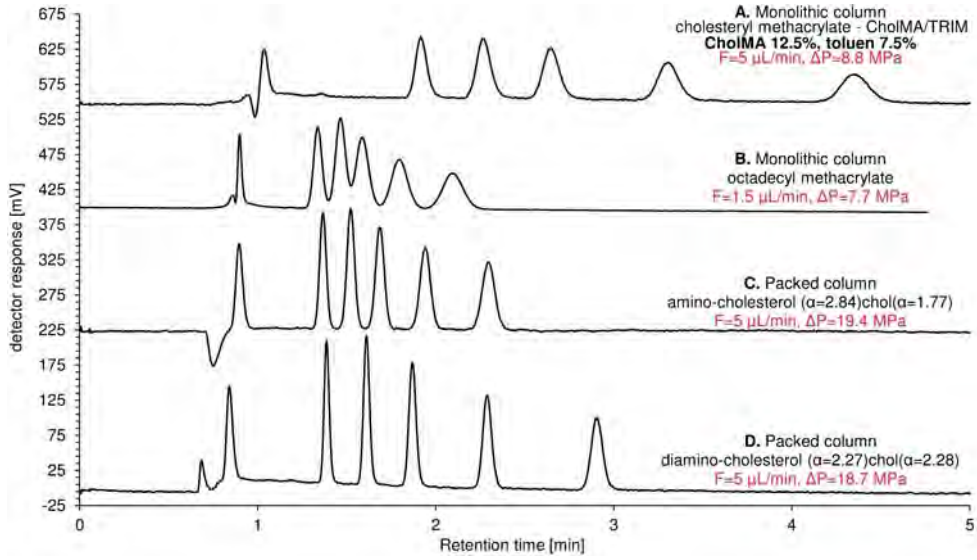


Fig. 2. The isocratic separations of alkylbenzenes on different stationary phases: (A) monolithic column CholMA/TRIM, (B) octadecyl monolithic column, (C) packed amino-cholesterol capillary column, and (D) packed diamino-cholesterol capillary column. Conditions: 30.5 cm × 180 µm i.d., mobile phase 80/20 ACN/H_2O; F = 5 µl/min and F = 1.5 µl/min, detection at 205 nm. Peaks resolved: thiourea, benzene, toluene, ethylbenzene, propylbenzene and butylbenzene in order of elution.

cholesteryl methacylte column were characterized by the longest retention times but also the lowest back pressure.

Cholesterol based stationary phases are known for their planar selectivity which is connected with the shape of a cholesterol molecule. A typical test mixture to assess the planar selectivity is that consisting of *o*-terphenyl and triphenylene. Fig. 3 demonstrates the isocratic elution of *o*-terphenyl/triphenylene mixture using 90/10 acetonitrile/water mobile phase. The silica-based amino-cholesterol material showed the highest resolution which was connected with its highest theroretical plate number and moderate selectivity (α = 3.41). The synthesized monolithic column showed the lowest resolution, which can be attributed to its poorer efficiency (broader *o*-terphenyl and triphenylene peaks), but the selectivity it provided was the highest (α = 3.97) in comparison with amino-Chol (α = 3.41) and diamino-Chol (α = 2.85). Additionally, octadecyl monolithic columns was not able separate this mixture.

3.2 Separations of large molecules compounds

Separations of alkylbenzenes is generally the first method to verify the efficiency of the column. However, the separation of large-molecular compounds would give better information regarding the resolution and efficiency of the column. In this case very good examples is separation of steroid hormones. In Fig. 4A we

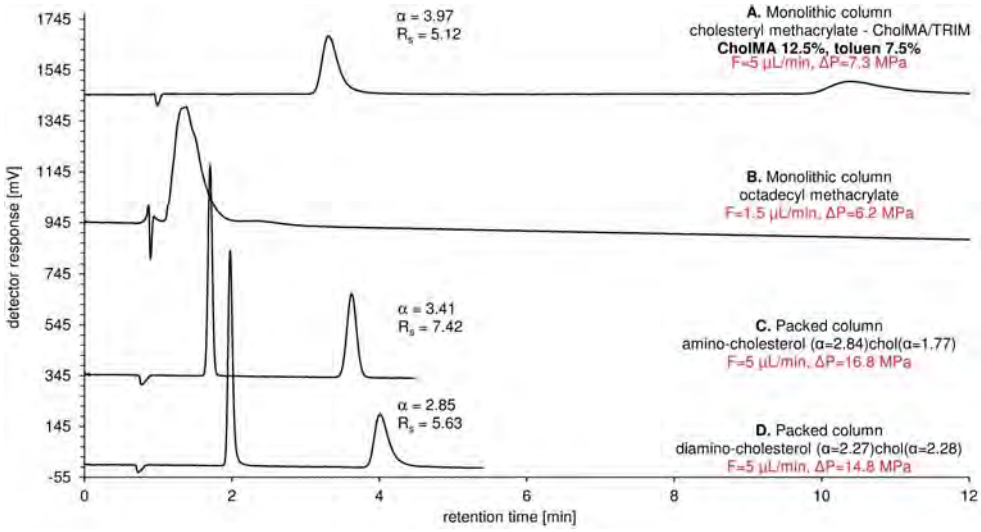


Fig. 3. The isocratic separations of *o*-terphenyl and triphenylene mixture (in order of elution) on different stationary phases: (A) monolithic column CholMA/TRIM, (B) octadecyl monolithic column, (C) packed amino-cholesterol capillary column, and (D) packed diamino-cholesterol capillary column. Conditions: 30.5 cm × 180 µm i.d., mobile phase 90/10 ACN/H_2O; F = 5 µl/min and 1.5 µl/min, detection at 254 nm.

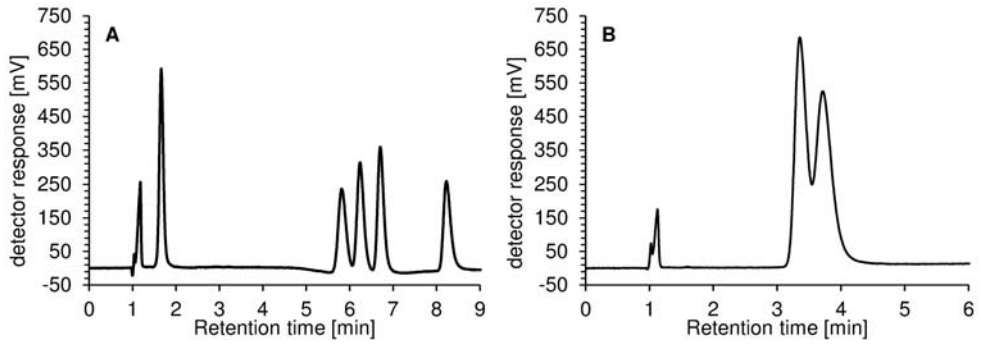


Fig. 4.

(A) Gradient separations of steroid hormones mixture. Conditions: 30.5 cm × 180 µm i.d. monolithic column; mobile phase component A was water, and B was acetonitrile; 60/40% – 2 min, and 30/70% from 2 to 10 minutes; F = 5 µl/min; detection at 222 nm; temperature 80 °C. Peak identifications: estriol, testosterone, estrone, β-estradiol, progesterone in order of elution.

(B) Isocratic separations of α- and β-estradiol. Conditions: mobile phase component A was water, and B was acetonitrile; 50/50%; F = 5 µl/min; detection at 222 nm; temperature 80 °C (from [19]).

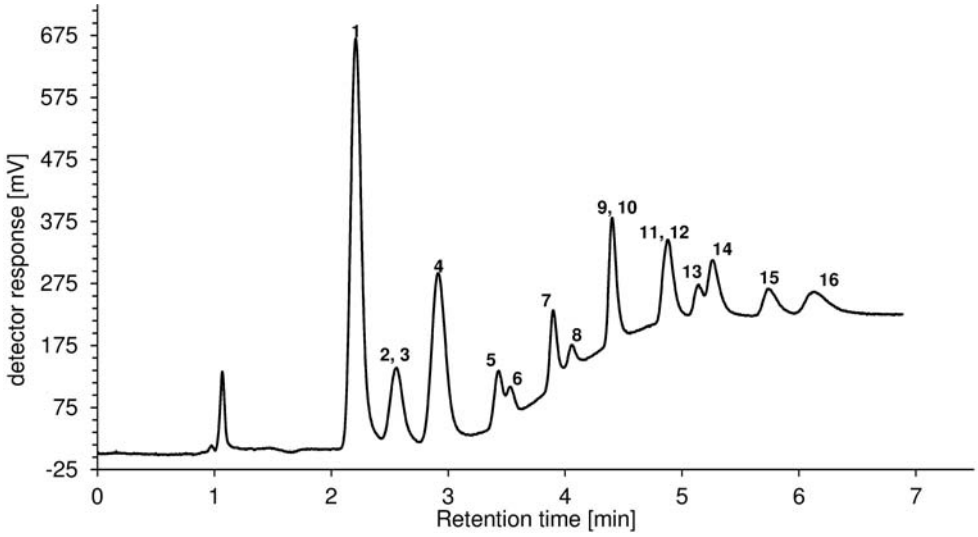


Fig. 5. Gradient separations of 16 polycyclic aromatic hydrocarbons mixture. Conditions: 30.5 cm × 180 μm i.d. monolithic column; mobile phase component A was water, and B was acetonitrile; linear A–B gradient from 75% to 100% B in 3 min, and then isocratic elution with 100% B; flow rate was 5 μl/min in the first 4 min, and then was 10 μl/min; on-column detection at 220 nm; temperature 100 °C. Peak identifications: (1) naphthalene, (2) acenaphtylene, (3) acenaphtene, (4) fluorene, (5) phenanthrene, (6) anthracene, (7) fluoranthene, (8) pyrene, (9) benzo[a]anthracene, (10) chrysene, (11) benzo[3]fluoranthene, (12) benzo[1]fluoranthene, (13) benzo[a]pyrene, (14) dibenzo[*a,h*]anthracene, (15) indeno[1,2,3-*c,d*]pyrene, (16) benzo[*g,h,i*]perylene (from [19]).

presented relatively fast separation of steroid hormones during 9 minutes. For this purpose, we used the gradient elution and performed the separation at 80 °C. The elevated temperature allowed to reduce retention times and improved symmetry of the peaks. Worthy of note is fact that, this monolithic cholesterol column provided significant stereoselectivity in the separation of α- and β-estradiol (Fig. 4B).

The Fig. 5, shows the chromatograms of separations of the 16 PAHs mixture using gradient elution of water (A) and acetonitriel (B) as a mobile phase. The flow rate was 5 μl/min during the first 4 minutes, then it was increased to 10 μl/min, while the mobile phase composition gradient was 75–100% B in 3 min. As it can be seen a successful separation of 13 PAHs was complete in less than 7 minutes. Taking into account the relatively low surface area of the monoliths (SBET=77.60 m^2/g, see our previous work [18]) this was a really good result.

4. Conclusions

New monolithic cholesterol-based stationary phase was synthesized using one-step thermal-initiated polymerization. These monolithic columns were successfully used for the separation of the low-molecular weight compounds (alkylbenzenes) and high-molecular weight compounds such as steroid hormones and PAHs. Additionally, this material showed good selectivity in separation of α- and β-estradiol.

Acknowledgments

The work was financed by the National Science Center (Krakow, Poland) grants no. 2013/11/N/ST4/01837. The financial support of the European Regional Development Fund under the ROP for the years 2007-2013: project no. RPKP.05.04.00-04-003/13 and Kujawsko-Pomorskie Voivodship Budget "Krok w przyszłosc" are also kindly acknowledged.

References

[1] Svec F. : *J. Chromatogr. A* **1217** (2010), 902.
[2] Hjertén S., Liao J.L., Zhang R.: *J. Chromatogr. A* **473** (1989), 273.
[3] Moravcova D., Jandera P., Urban J., Planeta J.: *J. Sep. Sci.* **27** (2004), 789.
[4] Guiochon G.: *J. Chromatogr. A* **1168** (2007), 101.
[5] Gore M.A., Karmalkar R.N., Kulkarni M.G.: *J. Chromatogr. B* **804** (2004), 211.
[6] Buszewski B., Jezierska M., Ostrowska-Gumkowska B.: *Mater. Chem. Phys.* **72** (2001), 30.
[7] Pesek J.J., Matyska M.T., Williamser E.J., Tam R.: *Chromatographia* **41** (1995), 301.
[8] Pesek J.J., Matyska M.T., Brent Dawson G., Wilsdorf A., Marc P., Padki M.: *J. Chromatogr. A* **986** (2003), 253.
[9] Al-Haj M.A., Haber P., Kaliszan R., Buszewski B., Jezierska M., Chilmonzyk Z.: *J. Pharm. Biomed. Anal.* **18** (1998), 721.
[10] Catabay A., Taniguchi M., Jinno K., Pesek J.J., Williamsen E.: *J. Chromatogr. Sci.* **36** (1998), 111.
[11] Soukup J., Jandera P.: *J. Chromatogr. A* **1245** (2012), 98.
[12] Buszewski B., Jezierska M., Wełniak M., Kaliszan R.: *J. Chromatogr. A* **845** (1999), 433.
[13] Soukup J., Bocian S., Jandera P., Buszewski B.: *J. Sep. Sci.* **37** (2014), 345.
[14] Buszewski B., Welerowicz T., Kowalkowski T.: *Biomed. Chromatogr.* **23** (2009), 324.
[15] Young J.E., Matyska M.T., Azad A.K., Yoc S.E., Pesek J.J.: *J. Liq. Chromatogr. Relat. Technol.* **36** (2013), 926.
[16] www.mtc-usa.com/wcode_udc.aspx
[17] www.nacalai.co.jp/english/cosmosil/column/20.html
[18] Szumski M., Grzywiński D., Buszewski B.: *J. Chromatogr. A* **1373** (2014), 114.
[19] Grzywiński D., Szumski M., Buszewski B.: *J. Chromatogr. A*., in press. DOI: 10.1016/j.chroma.2015.07.016.

Simple electrochemical DNA biosensor for detection of DNA damage induced by hydroxyl radicals

Andrea Hájková*, Vlastimil Vyskočil, Jiří Barek

Charles University in Prague, Faculty of Science, University Research Centre UNCE "Supramolecular Chemistry", Department of Analytical Chemistry, UNESCO Laboratory of Environmental Electrochemistry,
Hlavova 2030/8, CZ-12843 Prague 2, Czech Republic ✉ andrea.hajkova@natur.cuni.cz

Keywords
DNA biosensor
DNA damage
electrochemistry
glassy carbon electrode
hydroxyl radicals

Abstract
A novel simple electrochemical DNA biosensor based on a glassy carbon electrode was prepared by adsorbing low molecular weight double-stranded DNA from salmon sperm onto the electrode surface as a biorecognition layer. The biosensor was used for detection of DNA damage by hydroxyl radicals. Hydroxyl radicals are well-known reactive oxygen species inducing the oxidative stress in DNA. They can attack all the molecules, including DNA, and play a major role in the DNA oxidative damage. They were generated for our purposes electrochemically on the surface of a boron-doped diamond film electrode. A complex characterization of the specific damaging event was then obtained using a combination of several electrochemical detection techniques: cyclic voltammetry, square-wave voltammetry, and electrochemical impedance spectroscopy.

1. Introduction

In recent decades, detection of DNA damage has become one of the most important DNA research fields because of the critical role of DNA in mutagenesis, carcinogenesis, and aging. It is well known that DNA in biological systems can be damaged by variety of physical or chemical agents occurring in the environment, generated in the organisms as by-products of metabolism, or used as therapeutics. Therefore, detection of damage to DNA is of great importance for human health and its protection [1, 2]. Reactive oxygen species, such as superoxide radicals, hydroxyl radicals, hydrogen peroxide, and so on, can be generated in organisms via normal aerobic metabolism [3]. When the radical production is higher than the cellular antioxidant defense (oxidation stress), some radicals can cause cellular death, aging, and many diseases; such as cardiovascular disease, cancer, hepatitis, Parkinson's and Alzheimer's diseases [3, 4]. DNA biosensors, especially the electrochemical ones, represent sensitive, simple, rapid, and inexpensive tools to detect DNA damage [1, 2] and can thus be very useful in elucidation of the

mechanism by which DNA is oxidatively damaged by various reactive oxygen species.

2. Experimental

2.1 Reagents and chemicals

Different concentrations of low molecular weight salmon sperm double-stranded DNA (dsDNA; Sigma-Aldrich) were prepared by its dissolving in 0.1 mol L^{-1} phosphate buffer of pH = 6.7. The cyclic voltammetry and electrochemical impedance spectroscopy measurements were performed in 1×10^{-3} mol L^{-1} $[Fe(CN)_6]^{-4/-3}$ (Fe^{II}/Fe^{III}) in phosphate buffer, while the square-wave voltammetry measurements were carried out in pure phosphate buffer.

2.2 Instrumentation

Electrochemical measurements were carried out in a three-electrode system a platinum wire auxiliary electrode, a silver/silver chloride reference electrode (3 mol L^{-1} KCl), and glassy carbon electrode (GCE) or DNA biosensor based on a glassy carbon electrode (dsDNA/GCE) as a working electrode, and they were performed with a μAutolab III potentiostat.

The dsDNA/GCE was prepared by adsorption of salmon sperm dsDNA on the polished (using the aluminum oxide suspense) glassy carbon electrode. For its preparation, optimum parameters of the dsDNA adsorption were searched: a concentration of dsDNA, an adsorption potential (E_{ads}), and an adsorption time (t_{ads}). Optimum adsorption parameters were found to be: 0.1 mg mL^{-1} of dsDNA in phosphate buffer, E_{ads} = 0.5 V, t_{ads} = 30 s for square-wave voltammetry [2] and 10 mg mL^{-1} of dsDNA in phosphate buffer, E_{ads} = 0.5 V, t_{ads} = 180 s for cyclic voltammetry and electrochemical impedance spectroscopy.

The boron-doped diamond film electrode (BDDE) for generation of hydroxyl radicals was prepared by chemical vapor deposition, using a $CH_4/H_2/B_2H_6$ source gas mixture consisting of 1% of carbon with 10 ppm of B_2H_6 added for boron doping. The system pressure was 18.67 kPa, and the substrate temperature was 800 °C.

3. Results and discussion

Two detection modes were employed to detect DNA damage induced by hydroxyl radicals: (i) direct electrochemical method based on the oxidation of DNA bases (utilizing the square-wave voltammetry technique) [2] and (ii) indirect electrochemical method, using the DNA-specific redox active indicator Fe^{II}/Fe^{III} (utilizing the cyclic voltammetry and electrochemical impedance spectroscopy techniques) [5]. Square-wave voltammetry was carried out at the dsDNA/GCE biosensor

in phosphate buffer to monitor the changes in the intensity of the oxidation signals of guanine and adenine moieties before and after interaction with hydroxyl radicals. Cyclic voltammetry and electrochemical impedance spectroscopy were measured in Fe^{II}/Fe^{III} in phosphate buffer to monitor the changes in the height of the anodic and cathodic peaks of Fe^{II}/Fe^{III} and the changes in the charge transfer resistance. Hydroxyl radicals were generated electrochemically on the surface of the BDDE. The electrochemical oxidation process is described by following equation [6]:

$$BDDE + H_2O \rightarrow BDDE(^{\cdot}OH) + H^+ + e^- \quad (1)$$

Hydroxyl radicals were generated by the galvanostatic electrolysis on the BDDE in phosphate buffer with the dsDNA/GCE biosensor placed close to the BDDE surface (3 mm). The applied current density value plays a significant role in the electrolytic process. Therefore, the various current densities (5–50 mA cm^2) were examined. The changes on the dsDNA/GCE biosensor surface with increasing incubation time were monitored in pure phosphate buffer by square-wave voltammetry and in Fe^{II}/Fe^{III} in phosphate buffer by cyclic voltammetry and electrochemical impedance spectroscopy.

The square-wave voltammetry peaks of both guanosine and adenosine decreased in time, when the dsDNA/GCE biosensor was immersed into the solution containing hydroxyl radicals. At cyclic voltammetry, the increase of the anodic and cathodic peaks of Fe^{II}/Fe^{III} was observed together with the decrease of the charge transfer resistance value at electrochemical impedance spectroscopy, when damaged dsDNA released from the GCE surface. Initially native dsDNA was attacked and oxidized by hydroxyl radicals, which resulted in the release of nucleic acid bases and an interruption of the phosphodiester bonds [6]. In some cases, especially in the first stages of hydroxyl radical production, damaged dsDNA (nucleic acid fragments) formed a more compact film onto the GCE surface than the originally adsorbed dsDNA, and thus the electrode surface was more blocked for Fe^{II}/Fe^{III}.

4. Conclusions

A simple DNA biosensor was developed for complex electrochemical detection of damage to DNA by hydroxyl radicals whose generation was performed on the surface of the BDDE. Hydroxyl radicals interact with DNA bases and induce their damage. These radicals can attack and oxidize the DNA bases and deoxyribose, which leads to the formation of dsDNA strand breaks. Such structural changes can be successfully detected using the introduced dsDNA/GCE biosensor. Other alternatives to generate hydroxyl radicals are represented, e.g., by Fenton's reaction [7] or by electrolysis using lead dioxide film electrodes [8]. Both these options are planned to be involved in our forthcoming investigations, too.

Acknowledgments

This research was carried out in the framework of the Specific University Research (SVV 2015). Financial support from the Grant Agency of the Charles University in Prague (Project GAUK 430214/2014/B-CH/PrF) and from the Grant Agency of the Czech Republic (Project P206/12/G151) is gratefully acknowledged.

References

[1] Labuda J., Vyskocil V.: DNA/Electrode interface, detection of damage to DNA using DNA-modified electrodes. In: *Encyclopedia of Applied Electrochemistry.* Kreysa G., Ota K., Savinell R. (eds.). New York, Springer 2014, p. 346–350.
[2] Hajkova A., Barek J., Vyskocil V.: *Electroanalysis* **27** (2015), 101–110.
[3] Wang X., Chen R., Sun L., Yu Z.: *Int. J. Electrochem. Sci.* **9** (2014), 6834–6842.
[4] Gualandi I., Tonelli D.: *Talanta* **115** (2013), 779–786.
[5] Hlavata L., Benikova K., Vyskocil V., Labuda J.: *Electrochim. Acta* **71** (2012), 134–139.
[6] Oliveira S.C.B., Oliveira-Brett A.M.: *J. Electroanal. Chem.* **648** (2010), 60–66.
[7] Barroso M.F., Delerue-Matos C., Oliveira M.B.P.P.: *Food Chem.* **132** (2012), 1055–1062.
[8] Hmani E., Samet Y., Abdelhedi R.: *Diamond Relat. Mater.* **30** (2012), 1–8.

Analysis of low abundant lipids in vernix caseosa using chromatographic methods and mass spectrometry

Eva Háková[a, b, *], Radka Míková[a], Vladimír Vrkoslav[b], Richard Plavka[c], Josef Cvačka[b]

[a] *Charles University in Prague, Faculty of Science, Department of Analytical Chemistry, Hlavova 2030/8, 128 43 Prague 2, Czech Republic* ✉ *hakova@uochb.cas.cz*
[b] *Institute of Organic Chemistry and Biochemistry AS CR, v.v.i., Team of Mass Spectrometry, Flemingovo nám. 2, 166 10 Prague 6, Czech Republic*
[c] *Charles University in Prague, Department of Obstetrics and Gynaecology, General Faculty Hospital and 1th Faculty of Medicine, Apolinářská 18, 128 51 Prague 2, Czech Republic*

Keywords
gas chromatography
high performance liquid chromatography
mass spectrometry
vernix caseosa

Abstract
The aim of this work is to develop a separation method for characterization of unknown lipids in vernix caseosa. We focused on very low abundant lipids which have not been previously described in vernix caseosa. The total lipid extract was fractionated using low pressure normal phase liquid chromatography to obtain sub-fractions. The sub-fractions were further separated in high performance liquid chromatography mode to isolate classes of lipids. This procedure enabled us to characterise lipid classes by mass spectrometry and identify one of the lipid class as acyl-ceramides with non-hydroxylated fatty acids.

1. Introduction

Vernix caseosa is a multicomponent mixture, which is consisted of water (80 %) and proteins and lipids roughly in the same proportion (10 % each). This unique human material starts to be formed in the third trimester of a pregnancy and is present on the skin of newborns after a delivery [1, 2]. Vernix caseosa has a number of not fully understood functions. It works mainly to protect a fetus from the maceration in the amniotic fluid, protects a newborn during the birth from bacteria that populate the genital area of a woman and moisturizes the skin of the fetus [2]. It was found that vernix caseosa has a potential to be used in medicine because of its healing and antibacterial effects [3, 4].

The lipids of vernix caseosa are classified as barrier lipids (cholesterol, free fatty acids, phospholipids, ceramides) and nonpolar lipids such as sterol esters, wax esters and triacylglycerols which are originated from fetal sebaceous glands. These nonpolar lipids are also the main components of vernix caseosa lipids [5, 6].

Ceramides are the largest component of polar lipids in vernix caseosa. Nine types of ceramides have been identified so far using high performance thin layer chromatography in vernix caseosa and they were characterized by nuclear magnetic resonance and gas chromatography after their conversion to fatty acid methyl esters [7]. The molecular species of intact ceramides of vernix caseosa have not been described in detail yet. Rabionet et al. in 2003 published a presence 1-O-acylceramides with non-hydroxylated fatty acids in stratum corneum [8]. However, this type of ceramides has not been described in vernix caseosa, where other similar types of ceramides (α-OH-hydroxyacid/dihydrosphingosine ceramides) exist [7].

The present work describes a multidimensional separation of low abundant lipids. We hypothesise on the basis of the chromatographic behaviour, elemental formulae and composition of fatty acids after transesterification reaction that one of the investigated low abundant lipid sub-fraction corresponds to acyl-ceramides with non-hydroxylated fatty acids.

2. Experimental

2.1 Reagents and chemicals

Mass spectrometry grade methanol, hexane and propan-2-ol (Sigma-Aldrich) were used as received. Diethyl ether, chloroform (both from Penta, Czech Republic) and acetyl chloride (Fluka) were distilled in glass from analytical-grade solvents. Sodium methoxide was purchased from Sigma-Aldrich and silver carbonate was from Lachema (Brno, Czech Republic). Silica gel (10–100 μm, water content 10.3 %) was obtained from Merck and was activated according to Pitra et al. [9].

2.2 Sample collecting

Healthy male and female subjects delivered at full term were included in this study. Vernix caseosa samples (1–2 g) were collected immediately after the delivery into glass vials and stored at –25 °C. The exact location of sampling (back, buttocks, groins, legs, arms) varied depending on the vernix caseosa layer thickness. Blood contaminated samples were discarded. The samples were collected with written informed parental consent and the work was approved by the Ethics Committee of the General University Hospital, Prague (910/09 S-IV); the study was performed according to the Declaration of Helsinki.

2.3 Lipid extraction and separation of lipid classes

Lipids from vernix caseosa were extracted by chloroform:methanol (2:1, v/v). A large-scale separation of lipids was carried out using classical low pressure column chromatography with 4.7 grams of lipids isolated from vernix caseosa.

A silica gel column (length of glass column 41 cm, diam. 4.48 cm; particle size: 60–120 μm) with mobile phase hexane/diethyl ether gradient (from 1:99 to 50:50, v/v) was used to separate total lipid extract into fractions. The experiments were performed using LTQ Orbitrap XL hybrid FT mass spectrometer equipped with Ion Max source with ESI and APCI probe installed (Thermo Fisher Scientific) and coupled to HPLC, which was consisted of a Rheos 2200 quaternary gradient pump (Flux Instruments, Reinach, Schwitzerland), PAL HTS autosampler (CTC Analytics, Zwingen, Schwitzerland); the system was controlled by Xcalibur software (Thermo Fisher Scientific). The direct injection into ESI source was used for 30 fractions. The parameters of ESI source: the heated capillary temperature was set to 275 °C. Nitrogen served both as the sheath gas at a flow rate of 35 arbitrary units. The MS spectra of the positively charged ions were recorded from 150 to 2000 *m/z.* The sample of the fraction no. 23 was separated using Spherisorb column (250 + 250×4.6 mm, particle size: 5 μm; Waters) at 30 °C. The gradient program, phase A (hexane), B (hexane/propan-2-ol, 96:4, v/v): 0 min: 80% A/-20% B; 30 min: 71% A/29% B; 60 min: 53%A/47%B. The mobile phase flow rate was 1.0 mL/min and the injected volume of samples was 10 μl in each chromatography. The APCI vaporiser and heated capillary temperatures were set to 270 °C and 170 °C, respectively. Nitrogen served both as the sheath and auxiliary gas at a flow rate of 15 and 17 arbitrary units, respectively. The MS spectra of the positively charged ions were recorded from 250 to 2000 *m/z*.

2.4 Transesterification and GC/MS of fatty acid methyl esters

Lipids were transesterified using methods described by Stránský et al. [10] (for ester-linked fatty acids) and Oku et al. [7] (for amide-linked fatty acids). Fatty acid methyl esters were analyzed using an Agilent 6890N gas chromatograph coupled to a 5975B MSD quadrupole mass spectrometer and equipped with a fused silica capillary column Rxi-5ms (Restek). The carrier gas was helium at 1.0 mL/min. The injector was held at 230 °C and operated with a split ration 10:1; 2 μL of samples solution (hexan or chloroform:methanol (2:3, v/v)) was injected. The temperature program: 140 °C (0 min) to 330 °C (47 min); total run time was 47 min. 70 eV EI mass spectra were recorded in the mass range of 25–800 u; 4 min solvent delay was used. Temperatures of the transfer line, ion source and quadrupole were 280 °C, 230 °C and 150 °C, respectively. The chromatographic peaks representing fatty acid methyl esters were identified based on the presence of *m/z* 74 and *m/z* 87 in their mass spectra.

3. Results and discussion

The total lipid extract was separated into 30 fractions (mainly nonpolar lipids) by low pressure column chromatography. Due to the high complexity of the investigated material most of the fractions were mixtures as evident from TLC and

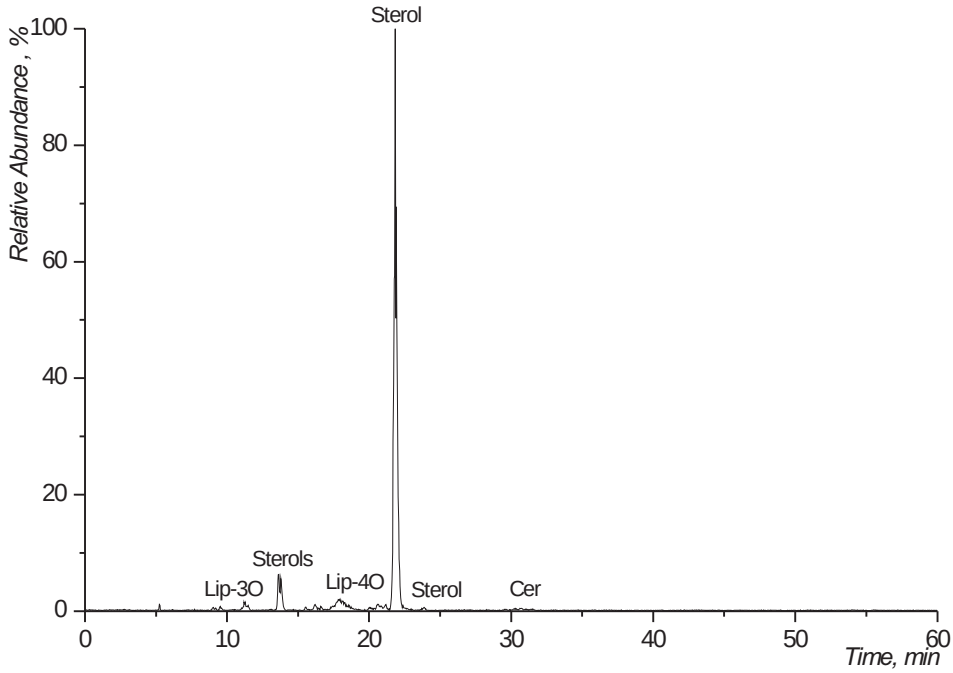


Fig. 1. NP-HPLC/MS base peak chromatogram of the fraction no. 23.

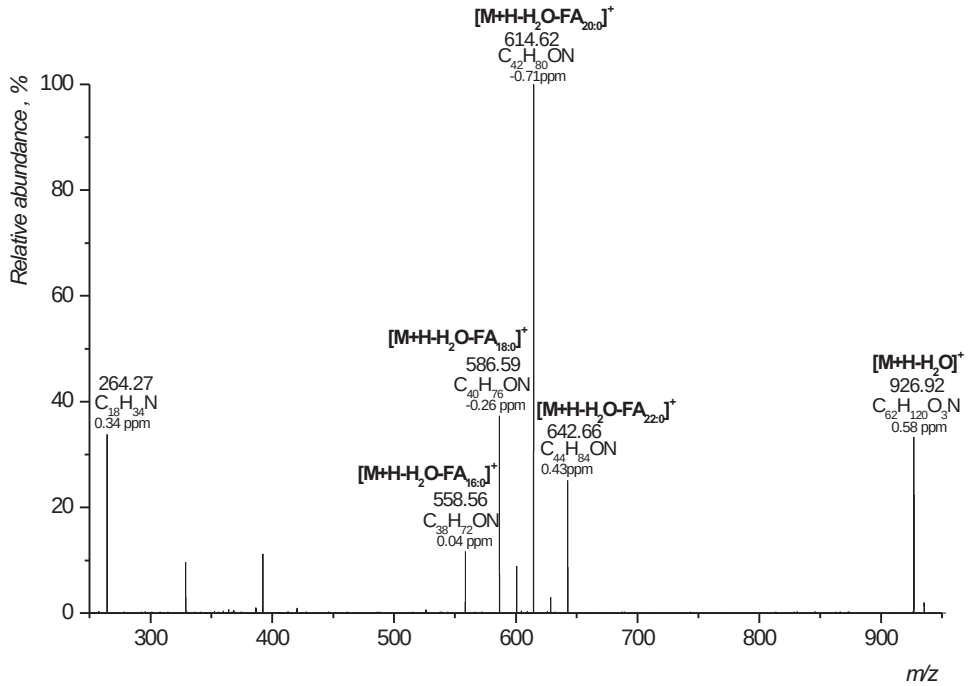


Fig. 2. APCI-MS/MS spectrum of dehydrated protonated molecule of an acyl-ceramide (M_{mi} = = 943.93 Da).

MS with direct infusion into the ESI source. In this work we focused on one particular fraction (F 23) containing several unknown lipid classes. In the next step we optimized high performance liquid chromatography method for separation of all lipid classes present in this fraction. The base peak chromatogram recorded under optimized chromatographic conditions is shown in Fig. 1; a good separation of individual lipid classes was achieved. The lipids were characterized using high resolution/accurate mass measurement mass spectrometry. General elemental formulas for lipid classes were obtained in this way. We detected lipids with three and four oxygens, sterols, and lipids with one nitrogen atom and four oxygens. We used this chromatographic system for a semi-preparative isolation of individual lipid classes and collected a sub-fraction of nitrogen-containing lipids. We hypothesized that these lipids could be structurally related to ceramides. The identification was supported by experiments based on transesterification reaction carried out in two different ways to cleave either ester-linked fatty acids or both ester and amide-linked fatty acids. Mainly saturated fatty acid methyl esters were observed in GC/MS data – for both procedures. An ESI-MS analysis of transesterifid samples made it possible to characterize remaining parts of the lipid molecules. We also employed tandem mass spectrometry and fragmented intact lipids (dehydrated protonated molecule $[M+H-H_2O]^+$, which was the spectrum base peak); the APCI-MS/MS spectrum is shown in Fig. 2. The ions at *m/z* 558.56, 586.59, 614.62 and 642.66 were rationalized as products of neutral losses of fatty acids (FA) from the parent ion, thus marked as $[M+H-H_2O-FA_{16:0}]^+$, $[M+H-H_2O-FA_{18:0}]^+$, $[M+H-H_2O-FA_{20:0}]^+$ and $[M+H-H_2O-FA_{22:0}]^+$, respectively. The other important peak *m/z* 264.27 was identified as dehydrated sphingosine. This fragmentised spectrum indicated presence of multiple isobaric species. These spectra also supported the hypothesis of acyl-ceramides with non-hydroxylated fatty acids.

4. Conclusions

Vernix caseosa is a biological material which is known for its high complexity. Therefore, the identification of new and low abundant components requires more separation steps. High resolution mass spectrometry makes it possible to determine elemental composition of the lipids in each chromatographic peak. The transesterification reaction helps to conclude the lipid structure because it brings detailed view of the composition of products of hydrolysis. The aforementioned method of analysis was used to discover and identify acyl-ceramides with non-hydroxylated fatty acids in vernix caseosa.

Acknowledgments

This work was supported by the Czech Science Foundation (Project No. P206/12/0750) and Charles University in Prague (Project No. SVV260205).

References

[1] Hoat S.B.: *Neonatal Skin, Structure and Function.* 2nd ed. New York, Marcel Dekker 2003.
[2] Pickens W.L., Warner R.R., Boissy Y.L., Boissy R.E., Hoath S.B.: *J. Invest. Dermatol.* **115** (2000), 875–881.
[3] Tollin M., Bergsson G., Kai-Larsen Y., Lengqvist J., Sjövall J., Griffiths W., Skúladóttir G.V., Haraldson Á., Jörnvall H., Gudmundsso G.H., Agerberth B.: *Cell. Mol. Life Sci.* **62** (2005), 2390–2399.
[4] Singh G.: *Indian J. Dermatol*. **53** (2008), 54–60.
[5] Kärkakäinen, J., Nikkari, T., Ruponen, S., Haahti, E.: *J. Invest. Dermatol.* **44** (1965), 333–338.
[6] Rissmann R., Groenink H.W.W., Weerheim A.M., Boath S.B., Ponec M., Bouwstra J.A.: *J. Invest. Dermatol.* **126** (2006), 1823–1833.
[7] Oku H., Mimura K., Tokitsu Y., Onaga K., Iwasaki H., Chinen I.: *Lipids* **35** (2000), 373–381.
[8] Rabionet M., Bayerle A., Marsching Ch., Jennemann R., Gröne H.J., Yildiz Y., Wachten D., Shaw W., Shayman J.A., Sandhoff R.: *J. Lipid Res.* **54** (2013), 3312–3321.
[9] Pitra J., Štěrba J.: *Chem. Listy* **56** (1962), 544.
[10] Stránský K., Jursik T.: *Eur. J. Lipid Sci. Technol.* **98** (1996), 65–71.

Determination of galactose and galactitol in urine by GC-MS/MS

Dušana Hudecová[a, *], Peter Kotora[a], Viktória Ferenczy[a, b], Róbert Kubinec[a], Darina Behúlová[b]

[a] *Comenius University in Bratislava, Faculty of Natural Sciences, Institute of Chemistry, Ilkovičova 6, 842 15 Bratislava, Slovakia* ✉ *samkova@fns.uniba.sk*
[b] *University Children's Hospital, Department of Laboratory Medicine, Centre of Inherited Metabolic Diseases, Limbová 1, 833 40 Bratislava, Slovakia*

Keywords
galactitol
galactose
galactosemia
GC-MS/MS

Abstract
A simple method for the determination of galactose and galactitol in urine samples using trimethylsilylation was developed. The developed method enables the analysis of urine samples without any other pretreatment except for derivatization. Urine samples of healthy controls and individuals with galactosemia were analysed. The limits of detection by GC-PCI-MS/MS were 2.68 mg L^{-1} for galactose and 2.70 mg L^{-1} for galactitol.

1. Introduction

Inherited metabolic diseases is the term applied to genetic disorders caused by loss of function of an enzyme or another specific protein. Enzyme activity may be low or lacking for variety of reasons. Some inherited metabolic diseases produce relatively unimportant physical features or skeletal abnormalities, but others produce serious diseases and even death [1].

Galactosemia was first "discovered" in 1908, when Von Ruess reported on a breast-fed infant with failure to thrive, enlargement of the liver and spleen, and "galactosuria" in publication entitled *Sugar Excretion in Infancy*. This infant ceased to excrete galactose through urine when milk products were removed from the diet. The toxic syndrome, galactosemia, is associated with an intolerance to dietary galactose as a result of certain enzymatic deficiencies [2].

Increased galactitol concentration is a common feature in galactokinase deficiency and has been implicated in galactosemic cataract formation. As conversion of galactose to galactitol by aldose reductase represents a dead-end metabolic pathway, galactitol removal is confined to renal excretion [2]. Classical galactosemia, galactose-1-phosphate uridyltransferase deficiency, an autosomal recessive disorder occurs in the population with an incidence of approximately 1:40–60,000. Galactose-1-phosphate, a metabolite derived from ingestion of galactose, is considered to be toxic in several tissues particularly in the liver, brain

and renal tubules [3]. Another type of galactosemia is a rare autosomal recessive disorder caused by UDP-galactose-4-epimerase deficiency.

Gas chromatography-mass spectrometry is the first choice approach for the measurement of monosaccharides [4, 5], as well as other small molecule metabolites in clinical settings [6]. However, a potential problem with GC-MS is the need of derivatization for polar and non-volatile analytes. The good volatility and stability characteristics of the derivatives formed make trimethylsilyl ethers the most popular derivatives applied to GC analysis of saccharides and polyalcohols. Several works have been published about the analysis of carbohydrates and polyalcohols in urine samples by different derivatization methods and their subsequent GC-MS analysis [7, 8–11].

The aim of this work was the development of a new, modified method enabling the simultaneous derivatization of galactose and galactitol in urine samples without any sample pretreatment before derivatization and their subsequent analysis by GC-MS/MS with positive chemical ionisation. The newly developed method was used for the analysis of the selected compounds in urine samples of healthy individuals and patients with galactosemia.

2. Experimental

2.1 Reagents and chemicals

BSTFA, galactose and galactitol were purchased from Sigma-Aldrich. Trifluoroacetic acid was purchased from Fluorochem Ltd. (Hadfield, UK). Acetonitrile and HMDS were bought from Merck. Urine samples were obtained from University Children's Hospital in Bratislava.

2.2 Instrumentation

The GC-MS/MS analyses were carried out with a Trace GC Ultra gas chromatograph equipped with a splitsplitless injector, a TriPlus autosampler and a TSQ Quantum XLS mass spectrometer (Thermo Fisher). One μL of the sample was injected into the injector operating in splitless mode (2 min). The temperatures of the injector and the MS-transfer line were 280 °C and 260 °C, respectively. Compounds were separated on a 30 m × 0.25 mm (i.d.) × 0.25 μm DB-5MS capillary column (Agilent Technologies, CA, USA) operating at constant helium flow of 1.5 mL min^{-1}. The column temperature was initially set to 80 °C, held for 1 min, which increased at a rate of 20 °C min^{-1} to 210 °C and then increased to 230 °C at a rate of 2 °C min^{-1}.

The transfer line to the ion source was held at 260 °C. The reagent gas used for positive chemical ionisation (PCI) was methane, introduced at a flow rate of 2.0 mL min^{-1}. The ion source temperature was 200 °C, the collision gas was argon with pressure of 1.5 Pa in the collision chamber. The main parameters related to

the mass spectrometer setup were ion source temperature 200 °C, electron energy 70 eV, emission current 50 μA. MS/MS detection was performed using SRM transitions experimentally optimized for the selected analytes. The used SRM transitions were *m/z* 361→169 for galactose with collision energy set to 15 V and *m/z* 345→255 galactitol with collision energy set to 15 V with a scan time of 50 msec each.

3. Results and discussion

Calibration standards of the analytes were prepared in synthetic urine according to Haglock-Adler et al. [12]. The limits of detection and other statistical parameters were calculated according to the EURACHEM guidelines [13]. The method was found to be linear in the concentration range 0.25–10,000 mg L^{-1} of the analytes. Limits of detection were 2.68 mg L^{-1} for galactose and 2.70 mg L^{-1} for galactitol.

For the derivatization of the analytes a two-step silylation method previously developed by Podolec et al. [14] was used. The use of strong silylation agents like BSTFA leads to the formation of multiple derivatives of easily silylable compounds, like sugars and sugar alcohols [15]. By the developed derivatization method only the expected derivatives of the analytes were formed and the silylation products otherwise susceptible to hydrolysis were stable in the presence of the aqueous matrix after derivatization. The developed method is therefore suitable for the determination of polar compounds in urine samples without the need of other sample pretreatment than derivatization.

Positive chemical ionisation is a soft ionisation method and therefore the fragmentation is not so extensive, higher fragments containing structural information are more abundant when compared to the most often used ionisation method EI. Tandem mass spectrometric detection is more selective than the usually preferred MS detection and thus makes possible the selective identification of analytes in a complex matrix without sample purification using the specific SRM transitions characteristic for the given compound. Fig. 1 shows a chromatogram of separation of the selected analytes using the developed GC-PCI-MS/MS method after sample derivatization. As can be seen from the chromatogram no sample impurities or other polar compounds present in the sample interfered with the analysis.

Real urine samples were obtained from the University Children's Hospital Bratislava. The samples were collected from four children with galactosemia at the age under one year, five galactosemic patients older than one year and fifteen healthy subjects in each age group. The concentration of galactose and galactitol in the samples was expressed both in mg L^{-1} and in μmol $mmol^{-1}$ creatinine. The values of creatinine concentration were obtained from the hospital laboratory. The concentration values of the analytes in selected urine samples (S1–S6) from galactosemic patients were listed in Table 1. As can be seen from the Table, the

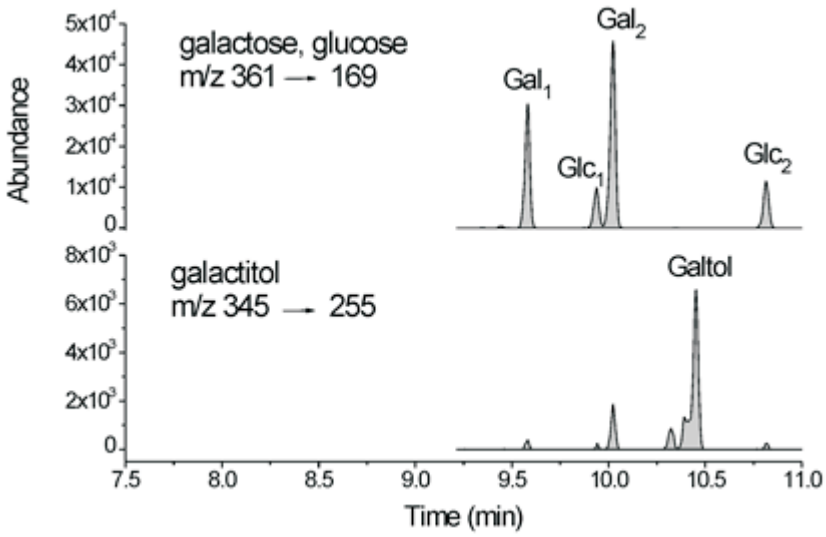


Fig. 1. GC-MS/MS chromatogram of trimethylsilyl derivatives of a urine sample from a galactosemic neonate. Peak identification in the reconstructed chromatogram: α-D-galactopyranoside (Gal_1), β-D-galactopyranoside (Gal_2), galactitol (Galtol), α-D-glucopyranoside (Glc_1), β-D-glucopyranoside (Glc_2).

the concentration of galactose and galactitol in urine was age-dependent, so the age of the patients should always be taken into consideration.

4. Conclusions

The developed method enables derivatization of galactose and galactitol directly in urine samples without the need of sample purification or other pretreatment. The GC-PCI-MS/MS method is selective with no interferences observed and with limits of detection sufficient for clinical analyses. The method is suitable for the detection of the selected analytes in urine samples and could be used for the diagnosis and lifelong follow-up of patients suffering from inherited disorders of galactose metabolism.

Acknowledgments

This publication is the result of the project implementation: Comenius University in Bratislava Science Park supported by the Research and Development Operational Programme funded by the ERDF. Grant number: ITMS 26240220086.

References

[1] Kavitha S., Sarbadhikari S.N., Ananth N.R.: *J. Helath Allied Sci.* **5** (2006), 2–6.
[2] Schadewaldt J., Killius S., Kamalanathan L., Hammen H.W., Strassburger K., Wendel U.: *J. Inherit. Metab. Dis.* **26** (2003), 459–479.
[3] Holton J.B., Walter J.H., Tyfield L.A. In: *The Metabolic and Molecular Bases of Inherited Diseases.* Scriver C.R., Beaudet A.L., Sly W.S., Valle D. (edits.). New York, McGraw-Hill 2001, p. 1553.

Table 1
Concentration of galactose and galactitol in urine samples of patients with galactosemia and healthy controls.

Age	Analyte	Sample						Control Samples			
		mg L^{-1}	µmol $mmol^{-1}$ of creatinine	mg L^{-1}	µmol $mmol^{-1}$ of creatinine	mg L^{-1}	µmol $mmol^{-1}$ of creatinine	mg L^{-1}	RSD (%)	µmol $mmol^{-1}$ of creatinine	RSD (%)
		S 1		S 2		S 3					
< 1 year	Galactose	764	3260	6030	22300	157	1090	75.2	147	411	103
	Galactitol	95.3	403	402	1470	66.8	460	30.3	104	132	27.1
		S 4		S 5		S 6					
> 1 year	Galactose	4.19	10.1	1.34	0.74	3.14	2.10	32.6	240	44.7	204
	Galactitol	85.9	205	316	173	312	207	50.2	102	52.9	65.8

[4] Biermann C.J. In: *Analysis of Carbohydrates by GLC and MS.* Biermann C.J., McGinnis G.D. (edits.). Boca Raton, CRC Press 1989, p. 2–17.

[5] Black G.E., Fox A.: *J. Chromatogr. A* **720** (1996), 51–60.

[6] Wajner M., de Moura D., Coelho R., Ingrassia R., Buker de Oliveira A., Brandt Busanello E.N., Raymond K., Pires R.F., Fischinger Moura de Souza C., Giuliani R., Regla Vargas C.: *Clin. Chim. Acta* **400** (2009), 77–81.

[7] Matsumoto I., Kuhara T.: *Mass Spectrom. Rev.* **15** (1996), 43–57.

[8] Xu K., Wang L., Cai H., Zhang T., Zhang C., Matsumoto I.: *J. Chromatogr. B* **758** (2001), 75–80.

[9] Kuhara T.: *J. Chromatogr. B* **781** (2002), 497–517.

[10] Schadewaldt P., Hammen H.W., Stolpmann S., Kamalanathan L., Wendel U.: *J. Chromatogr. B* **801** (2004), 249–255.

[11] Shoenmaker J.D.: *J. Vis. Exp.* **40** (2010) e2014, 1–3.

[12] Haglock-Adler C.J., Hurley A., Strathmann F.G.: *Clin. Biochem.* **47** (2014), 80–82.

[13] *EURACHEM Guide*. Teddington, LGC 1998.

[14] Podolec P., Hengerics Szabó A., Blaško J., Kubinec R., Górová R., Višňovský J., Gnipová A., Horváth A., Bierhanzl V., Hložek T., Čabala R.: *J. Chromatogr. B* **967** (2014), 134–138.

[15] Schummer C., Delhomme O., Appenzeller B.M.R., Wenning R., Millet M.: *Talanta* **77** (2009), 1473–1482.

Fluorescence spectroscopy for rapid classification of fruit spirits

Michaela Jakubíková*, Jana Sádecká

Slovak University of Technology, Faculty of Chemical and Food Technology, Department of Analytical Chemistry, Radlinského 9, 812 37 Bratislava, Slovakia ✉ *michaela.jakubikova@stuba.sk*

Keywords
fruit spirits
multivariate analysis
synchronous fluorescence spectrometry

Abstract
Fluorescence spectroscopy provides rapid profiling of food products and could become an effective tool for authentication when coupled to chemometrics. This study developed a simple method for classifying commercial fruit (apple, apricot, pear, and plum) spirits using synchronous fluorescence spectroscopy. Spectra were collected in the excitation wavelength range from 200 to 500 nm, with constant wavelength differences from 10 to 100 nm, and those obtained at wavelength differences 10, 90, and 100 nm were employed in multivariate analysis. Of samples, 100, 90, and 90% were properly classified by applying the linear discriminate analysis to the first principal components of the principal component analysis performed on the synchronous fluorescence spectra at wavelength differences 10, 90, and 100 nm, respectively. One hundred percent of samples were properly classified by applying the general discriminate analysis to spectra regardless of wavelength difference used.

1. Introduction

Fruit spirits are drinks produced exclusively by the alcoholic fermentation and distillation of fleshy fruit or must of such fruit, berries, or vegetables, with or without stones [1]. Freshly distilled spirits are subjected to a maturation process for periods of time that depend on traditional practices. They are usually aged in glass or earthenware containers, and hence are colorless. Well-known exception is Calvados, apple spirit aged in oak barrels, having amber color. Sometimes, distilled spirits made from other fruits, such as apricot, pear, and plum, are aged in wooden barrels and hence are colored [2, 3]. Another way of presenting fruit spirits is when the bottle contains a whole fruit inside.

Regarding a chemical composition, the major constituents of each fruit spirit are ethanol and water. Fresh distillates, which have not been aged in wood, are characterized by the presence of minor volatile compounds such as alcohols, aldehydes, esters, acids, volatile phenols, etc. Aging in wooden casks alters the chemical composition of fruit spirits, due to extraction of wood tannins, and chemical reactions such as hydrolysis of lignin and subsequent oxidation of benzoic and cinnamic aldehydes to aromatic acids [4–6]. A number of studies have been carried out to determine the volatile compounds composition of freshly

distilled or aged fruit spirits. Gas chromatography-mass spectrometry has revealed the presence of 167, 28, 55, and 107 volatile constituents in Calvados [7, 8], apricot [9], pear [10], and plum [11] spirits, respectively. The previous HPLC study has showed that phenolic acids could be present in wooden aged fruit spirits [3, 6, 12].

Various methods have been used to discriminate alcoholic beverages. The determination of relative levels of about 200 components in GC-MS analysis followed by a partial least squares discriminant analysis (PLS-DA) was suitable for distinguishing French-labeled brandies (Mirabelle, Calvados, Cognac, and Armagnac) [7, 8]. We have recently applied excitation-emission matrix and synchronous fluorescence spectroscopy for distinguishing between juniper-flavored spirit drinks. The best results were obtained with the principal component analysis-linear discriminant analysis (PCA-LDA) based on synchronous fluorescence spectra (SFS) [13]. The present study continues our effort to extend the possibility of application of fluorescence spectroscopy in classification of alcoholic beverages. Fluorescence spectra were used for characterization of commercially available apple, apricot, pear, and plum spirits. The aim of the study was to distinguish between four types of spirits by applying DA to the SFS of spirits.

2. Experimental

2.1 Samples

A total of 63 fruit spirits, representing four different fruits were analyzed: 15 apple spirits from four producers, 18 apricot spirits from four producers, 18 pear spirits from three producers and 12 plum spirits from four producers. 40 samples (eight apple, 12 apricot, 12 pear and eight plum spirits) were selected randomly for the calibration set, and 20 samples (four apple, six apricot, six pear and four plum spirits) for the prediction set. The alcoholic degree ranged within 35–42% ethanol.

2.2 Fluorescence spectroscopy

Fluorescence spectra were obtained using the Perkin-Elmer LS 50 Luminescence Spectrometer equipped with the Xenon lamp and quartz cell (10×10×45 mm). Excitation and emission slits were both set at 5.0 nm. The scanning speed was set at 200 nm min^{-1}.

Synchronous fluorescence spectra were collected by simultaneously scanning of the excitation and emission monochromators in the λ_{ex} range from 200 to 600 nm (step 1 nm), with constant wavelength differences $\Delta\lambda$ from 10 to 100 nm (step 10 nm) between them. Fluorescence intensities were plotted as a function of the λ_{ex}.

The spectrometer was interfaced to a computer supplied with FL Data Manager Software (Perkin-Elmer) for spectral acquisition and data processing.

2.3 Data Analysis

PCA was used to visualize the data trends. LDA and general DA (GDA) were applied as supervised learning method to find classification rules [14].

PCA is a method that aims to recognize patterns in multivariate data sets or to reduce the dimensionality of a data set by forming linear combinations of original variables called principal components (PCs). Classification of objects using PCA is done by constructing two- or three-dimensional plots, using PCs chosen by the researcher. The number of principal components was based on the eigenvalue criterion and the total variance explained [15].

LDA is concerned with determining the so-called discriminant functions as linear combinations of the descriptors which best separate the classes according to minimization of the ratio of within class and between-class sum of squares. LDA was based on data sets: the first PCs of the PCA performed on the SFS of bulk fruit spirits; PCA was used as tool for dataset size and co-linearity reduction [16]. The GDA approach was applied when the LDA did not produce a good classification. GDA was based on data sets: the SFS of bulk fruit spirits.

Data were exported to ASCII and processed with the Microsoft Office Excel 2010 software and STATISTICA version 7.0 (StatSoft, USA). Contour maps of fluorescence spectra were plotted using Windows-based software OriginPro 7.5 (OriginLab, USA).

3. Results and discussion

3.1 Fluorescence spectra

Total SFS of different types of bulk fruit spirits are presented in the form of contour maps. SFS of bulk colourless spirits showed the presence of two main excitation regions, namely, at about 250 and 300 nm, and contour maps spread in the excitation wavelength range 210–420 nm. SFS of bulk coloured spirits showed excitation maxima at longer wavelengths (260–270 nm and 320–350 nm), while contour maps spread in the excitation wavelength range 220–515 nm. The shape and intensity of SFS depended on the $\Delta\lambda$ value. The representative SFS for $\Delta\lambda$ = 10, 90 and 100 nm, which were employed in all subsequent calculations. In general, the spectra extended from 200 to 500 nm. Regarding $\Delta\lambda$ = 10 nm, SFS of apple spirits showed two bands in the wavelengths 275–422 nm with maxima at 302 and 395 nm. The first maximum was also observed for colourless apricot and plum spirits, while the second maximum for apricot spirit only. SFS of colourless pear spirits showed two maxima at shorter wavelengths, namely 291 and 323 nm. SFS of coloured spirits showed bands at various wavelengths. Thus survey information for different types of bulk fruit spirits is given in Table 1. In general, spectral features and fluorescence intensity values of all drinks are typical of drinks of similar origin and nature.

Table 1
Synchronous fluorescent properties of fruit spirit samples.

Sample	Colour	Δλ = 10 nm		Δλ = 90 nm		Δλ = 100 nm	
		Range [nm]	$\lambda_{ex,\,max}$ [nm]	Range [nm]	$\lambda_{ex,\,max}$ [nm]	Range [nm]	$\lambda_{ex,\,max}$ [nm]
Apple spirit	no	275–422	302, 395	211–400	220, 255, 302	200–404	222, 254, 301
Apricot spirit	no	281–410	304, 398	212–412	232, 309	216–416	228, 308
Apricot spirit with fruit	yes	215–479	263, 342	218–499	276, 347	216–476	265, 351
Apricot spirit with fruit	yes	276–487	342	254–507	342	281–489	341
Pear spirit	no	272–346	291, 323	200–324	224, 302	200–330	224, 302
Pear spirit with fruit	yes	221–479	260, 323	220–502	264, 340	220–483	262, 331
Pear spirit	yes	200–475	227, 338	220–501	231, 340	215–483	230, 341
Plum spirit	no	281–342	303	209–407	222, 263, 304	205–412	223, 260, 302
Plum spirit	yes	208–473	226, 321, 381	216–491	231, 329, 385	211–475	228, 329, 387

Table 2
Results of principal component analysis based on the synchronous fluorescence data (Egv = eigenvalue, Exv = explained variance).

PC	SFS Δλ = 10 nm			SFS Δλ = 90 nm			SFS Δλ = 100 nm		
	Egv	Exv [%]	Loading [nm]	Egv	Exv [%]	Loading [nm]	Egv	Exv [%]	Loading [nm]
1	25.7	64.3	260, 350	20.8	52.1	339	23.8	59.5	327, 387
2	8.4	20.9	305	14.3	35.8	220, 256, 303	11.8	29.6	223, 250, 300
3	2.5	6.3	294, 313, 400, 455, 478	3.0	7.5	220, 252	2.5	6.2	267, 254
4	1.7	4.4	291, 326	0.9	2.2	235, 276, 305, 354	1.0	2.4	235, 300, 352
5	0.9	2.2	259, 335	0.4	1.0	220,249,301, 324, 360	0.6	1.4	229, 271, 301, 325, 354
6	0.5	1.2	227, 304, 324	–	–	–	–	–	–
Total	39.7	99.4		39.4	98.6		39.7	99.4	

3.2 PCA, LDA, GDA

The results of PCA based on the synchronous fluorescence data are shown in Table 2. Applying PCA to SFS recorded at $\Delta\lambda$= 10 nm, the first two PCs explained the 85.2% of the total variance. PC1 explained 64.3% of the total variance, and the highest loadings were observed around 260 and 350 nm. PC2 explained 20.9% of the total variance. According to the loading, λ_{ex} = 305 nm was the most important variable in the PC2. The first two PCs lead to a good classification of group of pear spirits containing a whole fruit from group of others, based mainly on PC1. A group of colourless plum spirits was separated from group of others, based mainly on PC2. In general, the PCA score plot for the first two PCs indicates that it was not possible to discriminate between the different fruit spirits regardless of used.

LDA of the PC scores was used to improve discrimination between groups that were difficult to separate using PCA. LDA was carried out using the score values of the first PCs that gave the highest level of classification in the LDA. The inclusion of more PCs (from three up to six or five) when LDA models were developed improved the classification results. Regarding SFS recorded at λ_{ex} = 10 nm, the discrimination of the fruit spirits required six PCs (99.4% explained variation, Table 2) to achieve 100% of correct classification (Table 3) in both the calibration set and the prediction set. Performing LDA on five PCs of SFS obtained at λ_{ex} = = 90 nm or λ_{ex} = 100 nm the results become worst (total 90% of correct classification). Regarding λ_{ex} = 90 nm, 100% correct classification was observed for apple, apricot and plum spirit samples (Table 3), while only 67% of pear spirit samples were properly classified. A group of coloured pear spirits was classified as belonging to plum group. Regarding λ_{ex} = 100 nm, a group of colourless pear spirits was classified as apricot spirits.

Table 3
Percentage of correct classification of fruit spirits using LDA-PCA and GDA combined with synchronous fluorescence spectroscopy (t = training set, p = prediction set).

Sample	Set	SFS $\Delta\lambda$ = 10 nm		SFS $\Delta\lambda$ = 90 nm		SFS $\Delta\lambda$ = 100 nm	
		LDA-PCA	GDA	LDA-PCA	GDA	LDA-PCA	GDA
Apple	t	100	100	100	100	100	100
	p	100	100	100	100	100	100
Apricot	t	100	100	100	100	100	100
	p	100	100	100	100	100	100
Pear	t	100	100	67	100	67	100
	p	100	100	67	100	67	100
Plum	t	100	100	100	100	100	100
	p	100	100	100	100	100	100
Total	t	100	100	90	100	90	100

As the LDA classification based on PCs was unsatisfactory, with exception of PCs of the PCA performed on the SFS at λ_{ex} = 10 nm, we chose to discriminate the samples with GDA based directly on the SFS (λ_{ex} = 10, 90 and 100 nm) in the range of 200–500 nm with an interval of 5 nm. In this case 100% correct classification was observed for apple, apricot, pear and plum spirit samples (Table 3).

4. Conclusions

This study proved that the combination of SFS with multivariate statistical methods can be applied as a tool to classify commercially apple, apricot, pear, and plum spirits. It was also observed that the classification rates varied according to the classification method applied. Overall, LDA based on the first PCs did not achieve high classification rates as compared with GDA. 100% of samples were properly classified by applying the GDA to SFS regardless of wavelength difference used [17].

Acknowledgments

This research was supported by the Scientific Grant Agency of the Ministry of Education of Slovak Republic and the Slovak Academy of Sciences VEGA No 1/0051/13.

References

[1] European Union (EC) (2008) Regulation (EC) No. 110/2008 of the European Parliament and of the Council of 15 January 2008 on the definition, description, presentation, labelling and the protection of geographical indications of spirit drinks and repealing Council Regulation (EEC) No. 1576/89. OJEU L39 51 (2008), 1654.
[2] Tesevic V., Nikicevic N., Jovanovic A., Djokovic D., Vujisic L., Vučkovic I., Bonic M.: *Food Technol. Biotechnol.* **4** (2005), 367–372.
[3] Rusu (Coldea) T.E., Socaciu C.: *Bulletin UASVM Agriculture* **68** (2011), 518–528.
[4] Puech J.L.: *J. Sci. Food Agric.* **42** (1988), 165–172.
[5] Miller D.P., Howell G.S., Michaelis C.S., Dickmann D.I.: *Am. J. Enol. Vitic.* **43** (1992), 333–338.
[6] Mangas J., Rodríguez R., Moreno J., Suárez B., Blanco D.: *J. Agric. Food Chem.* **44** (1996), 3303–3307.
[7] Ledauphin J., Saint-Clair J.F., Lablanquie O., Guichard H., Founier N., Guichard E., Barillier D.: *J. Agric. Food Chem.* **52** (2004), 5124–5134.
[8] Ledauphin J., Le Milbeau C., Barillier D., Hennequin D.: *J. Agric. Food Chem.* **58** (2010), 7782–7793.
[9] Puškaš V., Miljíc U., Vasíc V., Jokíc A., Manovic M.: *Food Bioprod. Process* **91** (2013), 348–351.
[10] Versini G., Franco M.A., Moser S., Manca G.: *Int. J. Food Sci. Technol.* **47** (2012), 2519–2531.
[11] Velíšek J., Pudil F., Davídek J., Kubelka V.: Z. *Lebensm. Unters. Forsch.* **174** (1982), 463–466.
[12] Mrvcic J., Posavec S., Kazazic S., Stanzer D., Peša A., Stehlik-Tomas V.: *Croat. J. Food Sci. Technol.* **4** (2012), 102–111.
[13] Sádecká J., Uríčková V., Hroboňová K., Májek P.: *Food Anal . Methods* **8** (2015), 58–69.
[14] Coomans D., Massart D.L., Kaufman L.: *Anal. Chim. Acta* **112** (1979), 97–122.
[15] Kozak M., Scaman C.H.: *J. Sci. Food Agric.* **88** (2008), 1115–1127.
[16] Adams M.J.: *Chemometrics in Analytical Spectroscopy.* Cambrige, Royal Society of Chemistry 1995.
[17] Tomková M., Sádecká J., Hroboňová K.: *Food Anal. Methods* **8** (2015), 1258–1267.

Modification of QuEChERS followed by GC-MS for determination of pesticide residues in textile products

DOMINIKA JAROŠOVÁ *, AGNEŠA PÁLENÍKOVÁ, SVETLANA HROUZKOVÁ

Slovak University of Technology in Bratislava, Faculty of Chemical and Food Technology, Institute of Analytical Chemistry, Radlinského 9, 812 37 Bratislava, Slovak Republic
✉ *dominika.jarosova@stuba.sk*

Keywords
cotton
GC-MS
pesticide residues
QuEChERS
textile

Abstract
A new method using QuEChERS (quick, easy, cheap, effective, rugged and safe) followed by gas chromatography-mass spectrometry was developed for the simultaneous determination of 33 pesticide residues in cotton textile samples. The target on pesticides which are suspected or confirmed endocrine disruptors was given. Selection of extraction solvent and weight of sample were tested. Good linearity, satisfactory recoveries and repeatability were obtained. Limits of detection ranged between 0.02 and 14 μg kg^{-1} and limits of quantification between 0.06 and 47 μg kg^{-1}. The proposed method was applied to the analysis of real samples of T-shirts.

1. Introduction

Pesticide is a general term that includes a variety of chemical and biological products used to kill or control living organisms such as rodents, insects, fungi and plants. Even though pesticides play a significant role in agriculture, they belong to the most important environmental pollutants being present in water, soil, the atmosphere and agricultural products [1]. They have to be controlled due to their high toxicity and their widespread use. The concern has increased as certain pesticides and other synthetic chemicals may act as pseudo-hormones which disrupt the normal function of the endocrine system in humans and wildlife. This specific category of pollutants is known as endocrine disrupting chemicals or endocrine disruptors [2]. The European Commission defines endocrine disruptor as an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism or its progeny, or (sub)populations [3].

Cotton is one of the most important fibre and economic crops. It is planted in approximately 70 tropical/subtropical and temperate countries and more than 1300 insect species have been reported as cotton pest worldwide. The primary

approach to pest control is through the use of synthetic chemicals. More than 12 kg of pesticides per ha is required annually [4]. Pesticide residues in cotton may pose a risk to human health by skin contact. Therefore, it is necessary to develop a simple, rapid and sensitive method for their determination [5].

2. Experimental

2.1 Reagents and chemicals

Pesticide standards were obtained from different sources with purity >95% (Dr. Ehrensdorfer, Germany; Bayer, Germany; Cheminova, Denmark; Novartis, Switzerland; Agrovita, Slovak Republic). The solution of each of 33 pesticides was prepared in toluene at an approximate concentration of 1 g L^{-1}. The stock solution of 20 mg L^{-1} for all pesticides was prepared in acetonitrile. Working standard pesticide mixture solutions with lower concentrations were prepared in acetonitrile by dilution. Toluene and acetonitrile were high-purity-grade solvents for pesticide residues analysis Suprasolv® (Merck).

2.2 Instrumentation

GC-MS measurements were performed on an Agilent 6890N GC system coupled to an Agilent 5973N mass-selective detector (Agilent Technologies). The PTV was operated in the solvent vent mode under temperature programmed conditions. The flow through the split-valve was 50 mL min^{-1}. Chromatographic separation was performed using the following temperature program: 60 °C (hold 1.75 min), 60 °C min^{-1} to 150°C, 30 °C min^{-1} to 220 °C, 12 °C min^{-1} to 250 °C, 30 °C min^{-1} to 300 °C (hold 3 min). The total run time was 12.75 min. The injection volume was 2 µL. Helium with purity 5.0 (Linde) was used as a carrier gas in a constant flow mode at 1.2 mL min^{-1}. Narrow-bore chromatographic column CP-Sil 8 CB (Agilent Technologies) with 5% diphenyl 95% dimethylsiloxane stationary phase 15 m × 0.15 mm I.D. × 0.15 µm utilized was connected to a nonpolar deactivated pre-column (1 m × 0.32 mm I.D.; Supelco). The MS with EI mode (70 eV) was routinely set in the SIM mode, and for each pesticide, three specific ions were selected and sorted into groups. Dwell time was 10 ms. Retention times, target ions, qualifier ions and start times of SIM groups are given in Table 1.

3. Results and discussion

The selection of pesticides was performed utilizing the published information on pesticides applied to cotton fields or found in soil and water near the cotton fields [4–8]. Thirty-three pesticides belonging to different chemical classes (organochlorines, organophosphates, triazines, dinitroanilines, pyretroides and carbamates) and varying physicochemical properties were selected to fulfil the

Table 1
Retention times, target ions, qualifier ions and start times of SIM groups.

No.	Pesticide	t_r [min]	Monitored ions[a]			SIM group start time [min]
1	Carbofuran	4.191	**164**	149	131	3.00
2	Trifluralin	5.465	**306**	264	307	
3	Hexachlorobenzene	5.768	**284**	286	282	5.70
4	Dimethoate	5.873	**87**	93	164	
5	Atrazine	5.917	**200**	215	202	
6	Propazine	5.946	**214**	229	172	
7	Diazinon	5.974	**179**	137	152	
8	Lindane	6.002	**181**	183	109	
9	Parahtion-methyl	6.522	**263**	125	211	6.30
10	Promethrin	6.602	**241**	184	226	
11	Fenitrothion	6.740	**277**	125	260	6.70
12	Malathion	6.774	**173**	127	125	
13	Chlorpyrifos	6.854	**197**	314	199	
14	Aldrin	6.951	**263**	261	265	
15	Parathion-ethyl	7.008	**291**	109	139	
16	Dicofol	7.065	**139**	111	250	
17	Pendimethalin	7.197	**252**	162	281	7.16
18	Oxychlordane	7.340	**353**	355	115	
19	Heptachlor epoxide	7.345	**353**	355	351	
20	Bromophos-ethyl	7.505	**359**	357	303	
21	*o, p´*-DDE	7.591	**246**	248	318	
22	Endosulfan-alpha	7.764	**241**	239	195	7.70
23	*p, p´*-DDE	7.951	**246**	318	316	
24	Endrin	8.323	**263**	265	281	8.20
25	Endosulfan-beta	8.643	**237**	239	216	
26	*o, p´*-DDT	8.454	**235**	237	165	
27	*p, p´*-DDT	8.831	**235**	237	165	8.70
28	Endosulfan-sulphate	8.974	**229**	272	387	
29	Bifenthrin	9.203	**181**	165	166	
30	Methoxychlor	9.351	**227**	228	274	
31	Lambda-Cyhalothrin	9.700	**181**	197	208	9.50
32	Cypermethrin	10.706	**163**	165	181	
33	Deltamethrin	11.861	**181**	253	251	

[a] Qualifier target ions are marked in bold .

multiresidual character of the analysis. All the selected pesticides are suspected or confirmed endocrine disruptors.

3.1 Selection of extraction parameters

Various weight of sample (1, 2, 3, 5, 10 g) was tested, while using 10 mL of extraction solvent. The utilizable volume of extract (3 mL) was received using 1 g of sample.

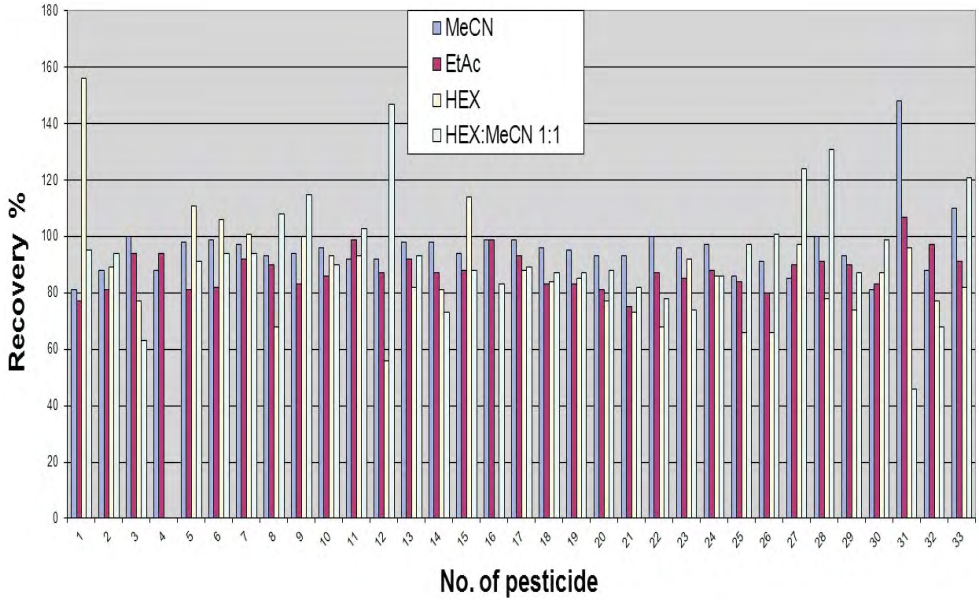


Fig. 1. Pesticide recoveries comparison depending on the solvent choice (numbers of pesticides correspond with numbers in Table 1).

Next study was focussed on the selection of extraction solvent. acetonitrile, ethyl acetate, hexane and the mixture of hexane:acetonitrile (1:1) were tested comparing the recoveries (Fig 1). Acetonitrile was selected as an extraction agent.

3.2 Method validation

To evaluate linearity, calibration curves were constructed. Matrix-matched standards were used to compensate the matrix effects. Eight concentration levels ranged from 0.1 to 250 μg kg^{-1}. Linearity of calibration curves constructed from absolute peak areas, expressed as R^2, was >0.97 for all pesticides, except metoxychlor and *p,p´*-DDT.

Instrument limits of detection and quantification were calculated by the MS software from the signal-to-noise ratio measured at the lowest calibration level. Signal-to-noise ratio values of 3 and 10 were used for the calculation of limits of detection and limits of quantification, respectively. Limits of detections were in the range of 0.02–14 μg kg^{-1} and limits of quantification were in the range of 0.06– 47 μg kg^{-1}.

Trueness of the analytical method was evaluated in terms of recovery by spiking blank cotton T-shirt. Recovery rates were evaluated at 1, 10, 50 and 250 μg kg^{-1} concentration levels. Recoveries ranged from 85 to 107% with RSD values ≤12.2%.

3.3 Real sample analysis

To demonstrate the applicability of the QuEChERS followed by GC-MS method the analysis of cotton T-shirt real samples was performed. The real samples did not contain the studied pesticides residues above the limits of detection.

4. Conclusions

Modified QuEChERS followed by GC-MS in EI mode was developed for determination of ultra trace levels of endocrine disrupting pesticide residues in cotton T-shirt matrices. It has provided high values of coefficient of determination for calibration measurements of matrix-matched standards. Recovery rates and further validation parameters at different concentration levels were determined. The method was applied to analysis of pesticide residues in real samples with no positive findings.

Acknowledgments

This work was supported by the Scientific Grant Agency of the Ministry of Education of the Slovak Republic and the Academy of Sciences (VEGA, project No. 1/0503/14) and by the Slovak Research and Development Agency under the contract No. APVV-0797-11. AP is also grateful for the Program for support of young researchers.

References

[1] Matisová E., Hrouzková S.: *Int. J. Res. Public Health* **9** (2012), 3166–3196.
[2] Andraščíková M., Hrouzková S.: *Anal. Methods* **5** (2013), 1374–1384.
[3] http://ec.europa.eu/environment/chemicals/endocrine/definitions/endodis_en.htm (accessed 18th June, 2015)
[4] Zhang B., Pan X., Venne L., Dunnum S., McMurry S.T., Cobb G.P., Anderson T.A.: *Talanta* **75** (2008), 1055–1060.
[5] Zhu F., Ruan W., He M., Zeng F., Luan T., Tong Y., Lu T., Ouyang G.: *Anal. Chim. Acta* **650** (2009), 202206.
[6] Cai J., Zhu F., Ruan W., Liu L., Lai R., Zeng F., Ouyang G.: *Microchem. J.* **110** (2013), 280–284.
[7] Abaga N.O.Z., Alibert P., Dousset S., Savadogo P.W., Savadogo M., Sedogo M.: *Chemosphere* **83** (2011), 585–592.
[8] Zhou X., Wang M., Sun Z., Li A., Xu L., Mu J., Lu L.: *J. Chromatogr. Sci.* **45** (2007), 375–399.

Kinetic study of rhenium complexes with 1,2,3-trihydroxybenzene

David Kaliba[a,*], Ivan Jelínek[a], Martin Štícha[b], Petra Vaňátková[a]

[a] *Charles University in Prague, Faculty of Science, Department of Analytical Chemistry, Hlavova 8, 123 48 Prague 2, Czech Republic* ✉ *david.kaliba@natur.cuni.cz*
[b] *Charles University in Prague, Faculty of Science, Department of Chemistry, Hlavova 8, 123 48 Prague 2, Czech Republic*

Keywords
kinetics aspects
mass spectrometry
rhenium
structural analysis
UV-Vis spectrometry

Abstract
Oxorhenium(V) complexes composed of rhenium isotopes and suitable ligands gain increased interest in radiopharmaceutical medicine. Selected rhenium complexes with 1,2,3-trihydroxybenzene as ligand were prepared by a reaction of rhenium(+V) precursor (tetrabutylammonium tetrachlorooxorhenate) with twofold molar excess of ligand in acetonitrile. The structure of formed complexes and their degradation products were obtained by mass spectrometry with electrospray ionization and else verified with nuclear magnetic resonance and infrared spectroscopy. The kinetics of reactions occurring in course of complex formation and subsequent degradation were followed by electrospray mass spectrometry and UV/VIS spectrometry.

1. Introduction

Present nuclear medicine frequently uses transition radiopharmaceuticals injected to patient's body in form of metal radionuclide ion and organic ligand complexes [1]. Primary and secondary tumors can be diagnosed by a suitable radio imaging technique. Diagnostics methods compare changes in metabolism of pathological lesion and surrounding tissue in a completely non-invasive way. Detailed knowledge of the composition of complex forming reaction mixture, namely the structure of final complexes and their degradation products and kinetic aspects of transitions between individual complexes are important before application to patient body.

Oxorhenium(V) complexes composed of β emitting rhenium isotopes (^{186}Re and ^{188}Re) and complex forming ligand play important role in radiopharmaceutical medicine because of their similarity with already in medicine used technetium analogues. Coordination compounds of ^{99m}Tc are well established in nuclear diagnostics; among other radionuclides, there is considerable research interest in ^{186}Re and ^{188}Re for applications in bone antitumor therapy. Both metals possess almost identical chemical properties [2, 3]. They show in their compounds a wide variety of oxidation states ranging from (−I) to (+VII) and form both

cationic and anionic species with strong oxidizing to mild reducing properties. As other transition metals, they are able to create coordination complexes with inorganic and organic ligands, the stability of whose depend on individual redox states of central metal ion and coordinated ligands. For rhenium based radiopharmaceuticals, defined reduction of perrhenate and its stabilization via suitable complex formation still remain crucial breakthroughs waiting to be achieved.

There is a lack of exact analytical information describing individual chemical reactions of rhenium precursor with a ligand and other components present in reaction mixture. Also relevant data about stabilities various rhenium complexes and their possible decomposition routes are missing. Several kinetics studies have been reported as a support for the description of catalytic properties of transition metal complexes. Ligand exchange of carbon monoxide and substitution reactions of $Mn(CO)_5X$ and its derivatives have been described [4–6]. At relatively high temperature rhenium pentacarbonyl halides, $Re(CO)_5X$, react with several monodentate ligands having donor atoms (phosphorus, nitrogen, sulphur) to form disubstituted compounds $Re(CO)_3XL_2$ [7]. Pyridine, γ-picoline and α-chloropyridine were used as forming ligands to reaction with rhenium precursor and kinetic studies were monitored by infrared spectrometry [8]. Kinetic addition of nucleophiles, pyridine *N*-oxides, triarylphosphates, 2,7-nonadiyne and glucose to rhenium was monitored in vitro and in vivo and structure investigations were done [9–13]. Rhenium core can be also used as catalyst to multielectron atom or oxygen transfer reaction of perchlorate, dithiolate complexes, epoxides, glycols, olefins epoxidation or in ammonia synthesis [14–19].

Rhenium complexes are usually characterized by common analytical methods, selected on the basis of desired information; the changes of rhenium oxidation states are monitored by means of electro-analytical and spectrometric methods [20, 21], quantification of the components in reaction mixtures is achieved by means of thin layer chromatography, high performance liquid chromatography and capillary zone electrophoresis or alternatively [22–24]. Mass spectrometry, nuclear magnetic resonance and infrared spectrometry are used for the purposes of structure characterization of the products of complex forming reactions.

Mass spectrometry found its irreplaceable role in modern instrumental analytical chemistry, making it possible to evaluate structure of analytes in mixtures and in trace concentrations. Structure characterization of organometallic complexes utilizes common methods of instrumental analysis, such as X-ray diffractometry in combination with nuclear magnetic resonance and infrared spectrometry [25, 26]. These methods are suitable for identification of pure reaction products and, in principle, less applicable for the evaluation of their structural transformations in solutions, e.g. for monitoring of reaction kinetics in course of their preparation and stability studies.

Mass spectrometry with soft ionization techniques seems to be suitable method for the analysis of various complexes. Electrospray ionization (ESI) usually provides mass spectra with dominant molecular ion and minor fragment

ions [27]. As compared with NMR and IR techniques, ESI-MS makes it possible to achieve substantially lower detection limits and is applicable to analytes in aqueous solutions [28–30]. Similarly, laser induced photo desorption (LDI) and matrix assisted photo desorption (MALDI) proved to ionize effectively the complexes providing mass spectra with dominant molecular ions and low extent of fragmentation [31, 32]. Atmospheric pressure photoionization (APPI) is, at present, the softest commercially available ionization technique that was successfully utilized for structure characterization of complexes [33]. Low energy UV lamp excitation light enables to generate intact molecular ions for kinetically labile analytes with negligible extent of fragmentation. The comparison of the analysis of selected rhenium(+I) complexes by means of LDI, MALDI and ESI ionization can be found in ref. [34, 35].

2. Experimental

2.1 Reagents and chemicals

Tetrabutylammonium-tetrachlorooxorhenate (n-Bu_4N)[$ReOCl_4$] was purchased from Aldrich, 1,2,3-trihydroxybenzene (pyrogallol) was purchased from Alfa Aesar, acetonitrile (ACN-d3) for NMR 99.8% purity was purchased from Euro--isotop, acetonitrile (HPLC grade) was purchased from Lach-Ner (dried and deoxygenated before use if necessary), trietylamine (TEA) was purchased from Sigma, ESI tuning mix for Ion trap was purchased from Agilent. All other reagents used to the synthesis were commercially available and were used without further purifications.

2.2 Instrumentation

ESI-MS experiments were conducted on an ion trap instrument Esquire 3000 (Bruker Daltonics, Germany). Proton and ^{13}carbon nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra were run in deuterated acetonitrile (ACN-d3) and recorded using Bruker Avance III (300 MHz) NMR spectrometer. Infrared spectra of prepared complexes were recorded on NICOLET 380 FT-IR using KBr discs. UV/VIS absorption spectra were measured by Thermo Evolution 60 using 3 mL glass or plastic cuvettes with defined 1 cm length.

2.3 Preparation of bis(1,2,3-trihydroxybenzene)oxorhenium complexes

Methods of preparation rhenium complexes with pyrogallol are shown in Fig. 1 (in curly braces are show different reaction ways) including reaction conditions and reaction time for every degradation or storable reaction in schema and oxidation state of rhenium atom in complex. Obtained masses of prepared complexes by mass spectrometry are given in Table 1.

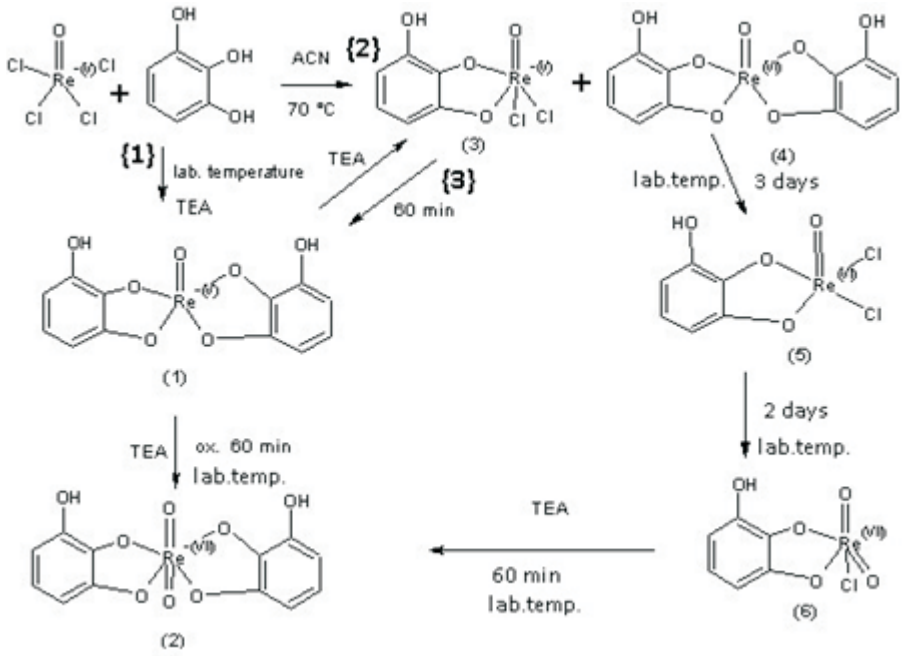


Fig. 1. Scheme of reactions between rhenium precursor (n-Bu_4N)[$ReOCl_4$] and 1,2,3-trihydroxybenzene as complex forming ligand. Conversion reactions between compounds, reaction conditions and rhenium oxidation states. Observed reaction pathways are numbered in curly braces.

Table 1
Formulas and molecular masses of rhenium complexes observed and identified in negative ESI/MS spectra.

Entry	Formula	*m/z*
(**1**)	$ReC_{12}H_8O_7$	451
(**2**)	$ReC_{12}H_9O_8$	467
(**3**)	$ReC_6H_4Cl_2O_4$	397
(**4**)	$ReC_{12}H_8O_8$	450
(**5**)	$ReC_6H_4Cl_2O_4$	397

Method **{1}**: The reaction of (n-Bu_4N)[$ReOCl_4$] (0.88 mg = 1.42 μM) with pyrogallol (0.48 mg = 3.81 μM) in deoxygenated or untreated acetonitrile (3 ml) was performed and after 10 min a volume of 11–63 μL TEA (in molar excess in deoxygenated acetonitrile) was added. Resulting yellow colored solution with prevailing complex $[Re(O)(PG)_2]^-$ (**1**) gradually changed its color to brown as it was converted to complex (**2**).

Method **{2}**: Mixture of (n-Bu_4N)[$ReOCl_4$] (0.88 mg = 1.42 μM) and pyrogallol (0.48 mg = 3.81 μM) in deoxygenated or untreated acetonitrile (3 ml) was heated on oil bath (70 °C) for 40 min. The resulting purple solution containing major complexes (**3**) and (**4**) was cooled and after 60 min 5 μL (= 35.8 μM) TEA was added to reaction mixture. Within 60 min, yellow solution with prevailing complex (**1**) changed its color to brown (dominant (**2**)) in 60 min. This solution

with major compound (**2**) was used for UV/VIS, IR, NMR and MS measurements described below.

Method **{3}**: The mixture of complexes (**3**) and (**4**) (products of method {**2**}, before addition of TEA) was left at room temperature for 5 days. Within 3 days colored change from purple to blue was observed as the content of complex (**5**) increased. Consequently, blue solution changed its color to green with dominating complex (**6**).

NMR and IR supplementary data: Complexes (**1**), (**2**) and (**4**) are almost identical for NMR experiments and provides shifts at (600 MHz, CDCN): δ: 9.95 (bs, 2H), 6.57–6.55 (m, 2H), 6.35–6.34 (m, 4H). Characteristic shifts of terabutylammonium cation at 3.09–3.06 (m, 8H), 1.62–1.57 (m, 8H), 1.37–1.33 (m, 8H), 0.97 (t, j=7.3 Hz, 12H) ppm are common for all complexes. Complex (**5**) gives shifts at (300 MHz, CDCN): δ: 7.27–7.25 (2H), 2.02–2.01 (2H) and 0.085–0.075 (1H). Shifts at (300 MHz, CDCN): δ: 7.07–7.01 (2H), 5.38–5.34 (8H) and 5.23–5.18 (2H) were measured for complex (**6**). In IR spectra of prepared complexes there is a strong absorption in the region 910–1000 cm^{-1} assigned to a characteristic stretch of terminal Re=O group. The typical intense aromatic C=C stretching vibrations give rise to absorption bands in the region 1470–1520 cm^{-1}. Intense absorption bands at 2962, 2932 and 1509 cm^{-1}, respectively correspond to the presence of tetrabutylammonium counter cation.

2.4 Method of mass spectrum analysis

In ESI-MS analysis the nebulizer gas pressure was 18 psi. In all measurements the flow rate of dry gas was as set 5 L/min and the temperature was 250 °C. The sample was diluted in acetonitrile and delivered to nebulizer by a syringe pump (Cole Parmer, USA) at a flow rate at 8 μL/min. Optimized conditions of mass spectrometer in negative ion modes were as follows: capillary voltage was 4000 V, flow rate of desolvation gas was as set 5 L/min, desolvation temperature was 250 °C.

2.5 Kinetics studies of complexes transitions

Kinetic conditions of individual complexes transitions were followed by mass spectrometry with electrospray ionization and compared with those obtained by UV/VIS spectrometry. ESI spectra for ESI kinetic were measured with: capillary voltage 4000 V; nebulizer gas pressure 15 psi, dry gas flow 4,5 L/min at temperature 300 °C, scan mode 110–500 *m/z* with scan speed 5,500 *m/z* per sec.; syringe pump flow 8 μL/min. The instrument was controlled and manipulated through the Esquire Control 5.3.11 software and experimental data were processed via Data Analysis 3.3.56. UV/VIS spectra were measured in quartz 3 mL cuvettes with defined 1 cm optical length using following experimental condition: scan 250–700 nm, interval 1 nm, threshold 0,01 A, measurement mode A

(0–4 mAU), wait time 0 s, total time 60–12,000 min, interval time 60 s. UV/VIS kinetic measurements were performed in time set on 60 min under condition mentioned above.

3. Results and discussion

Described preparation procedures of rhenium complexes with 1,2,3-trihydroxybenzene as complex forming ligand and rhenium precursor result in different color of resulting solutions. As it is evident from reaction scheme in Fig. 1, two different ways (**{1}** and **{2}**) of preparation in deoxygenated acetonitrile yield identical structures of final complexes (**1**) and (**4**) with two ligands attached to the rhenium core, differing only in oxidation state of rhenium. In yellow solution (product of method **{1}**), complex (**1**) with rhenium in oxidation state +V and with one oxygen atom bounded to the core was identified. This complex is unstable and after addition triethylamine to solution it is easily converted to complex (2) with two ligands and two oxygen atoms attached to the rhenium core in oxidation state (+VII). This reaction is reversible, after addition triphenylphosphine to brown reaction mixture one oxygen atom is eliminated and oxidation number of rhenium returns to +V (compound (**1**)).

The second procedure (method **{2}**) of complex preparation led to purple colored solution with prevailing compounds (**3**) and (**4**) with one or two ligand molecules attached to the rhenium core, respectively, differing in oxidation number of rhenium. In complex (**3**) one ligand, two chlorine atoms and one oxygen atom are bound to the core. In complex (**4**) chlorine atoms are not present however, oxygen atom with two molecules of ligand are bound to rhenium ion in oxidation state +V. After addition triethylamine to solution complex (**4**) is easily converted to compound (**1**). Transition between complexes (**3**) and (**4**) and complex (**1**) is fully reversible and depends only on addition of triethylamine.

Kinetic velocity of individual transitions as well as concentration increasing and decreasing of compounds in reaction mixture was measured by mass spectrometry and compared with those from UV/VIS spectrometry. Due to the presence of O-substituted aromatic ligands, the complexes studied in this work show strong absorption in the UV/VIS region. Time-bases ESI/MS measurements enabled to specify different reaction pathways yielding different rhenium complexes. From Fig. 2 it is evident, that complex (**2**) is a final oxidation reaction product of primarily formed complex (**1**). The rate of this reaction significantly depends on the residual content of oxygen in acetonitrile. As it is evident from Fig. 2A and 2B, the formation of complex (**2**) is significantly promoted by the presence of oxygen (measurements were performed in deoxygenated or untreated acetonitrile with or without air access). Interconnection of alerts describing relative mass intensity for complexes (**1**) and (**2**) for measurement achieved in untreated acetonitrile (part 3B) incoming in 25th min indicates faster conversion reaction between complexes because of air access. Without air access (part 3A) conversion reaction

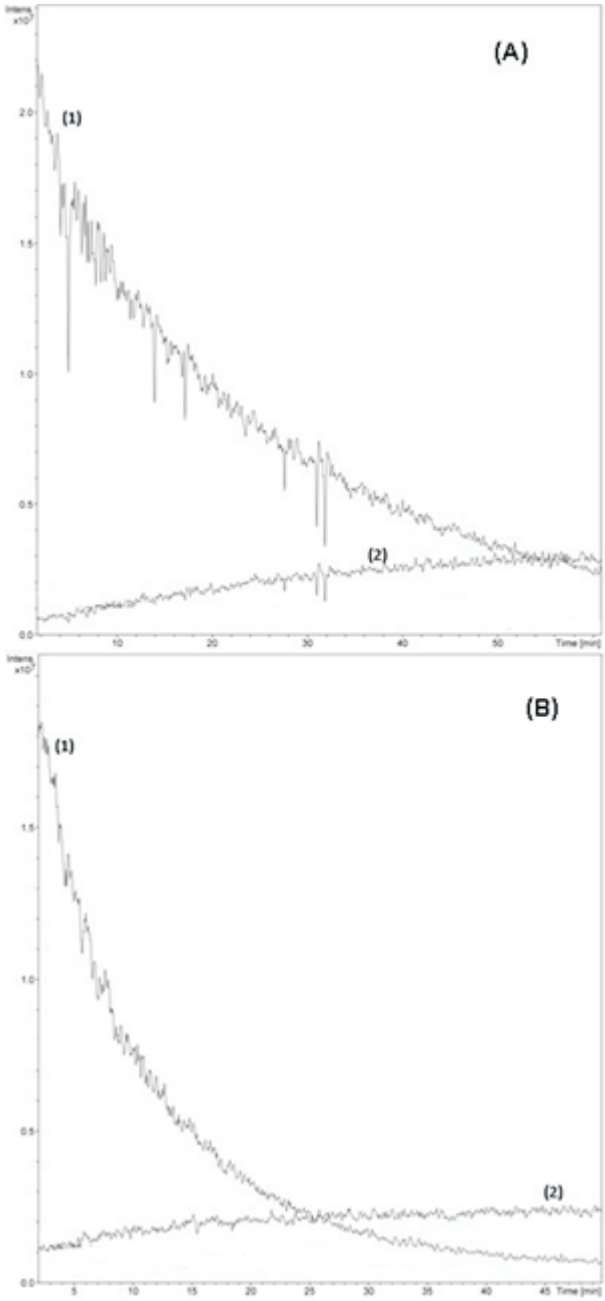


Fig. 2. ESI-MS kinetic dependencies of transition reaction between complexes (**1**) and (**2**) in deoxygenated ACN (A) and untreated ACN (B). Time of measuring was set to 60 min.

is significantly slower and interconnection arises in 55th min. In contrast to complex (**1**), complex (**2**) containing rhenium ion in oxidation state (+VII) is a product stable on air and in aqueous solutions.

Differences in colors between complex (**1**) and complex (**2**) enable to monitor above mentioned conversion by UV/VIS spectrometry (Fig. 3). Decrease of absorption maximum at 270 nm corresponds to the decrease of the concentration of Re(+V) precursor in course of the reaction. The formation of complex (**1**) is documented by an increase of absorbance at 430 nm, consequent oxidation to complex (**2**) is evident from an increase of the absorbance at 575 nm connected with proportional decrease of absorption maximum at 430 nm. As ESI kinetic measurements proved, reactions without access of oxygen were significantly slower than reactions with oxygen access. Therefore, time set on 60 min for measurement was not enough for completion of conversion reaction and only one absorbance maximum (at 575 nm) was obtained in reaction performed in deoxygenated acetonitrile.

The rate of complex forming reaction yielding complex (**2**) depends significantly on the addition of triethylmine as a base binding Cl^- ions as reaction by-product. As it is evident from kinetic curves shown in Fig. 4, the rate of reaction significantly increases with increasing molar excess of triethylamine in reaction

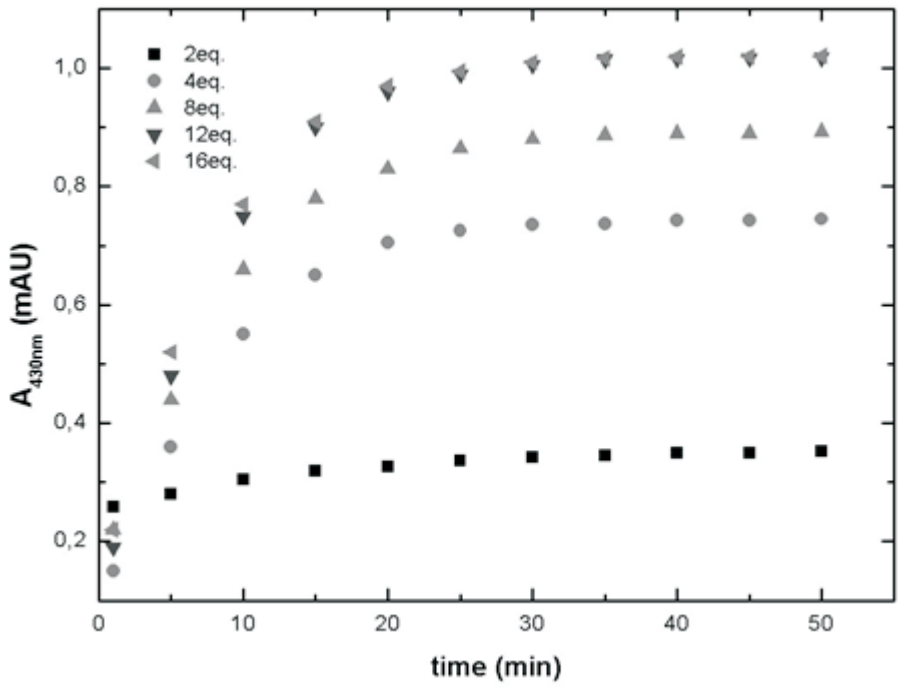


Fig. 4. The dependencies of the yield of final complex (**2**), expressed as an increase of absorption maximum at 430 nm, on molar excess of triethylamine in reaction mixture.

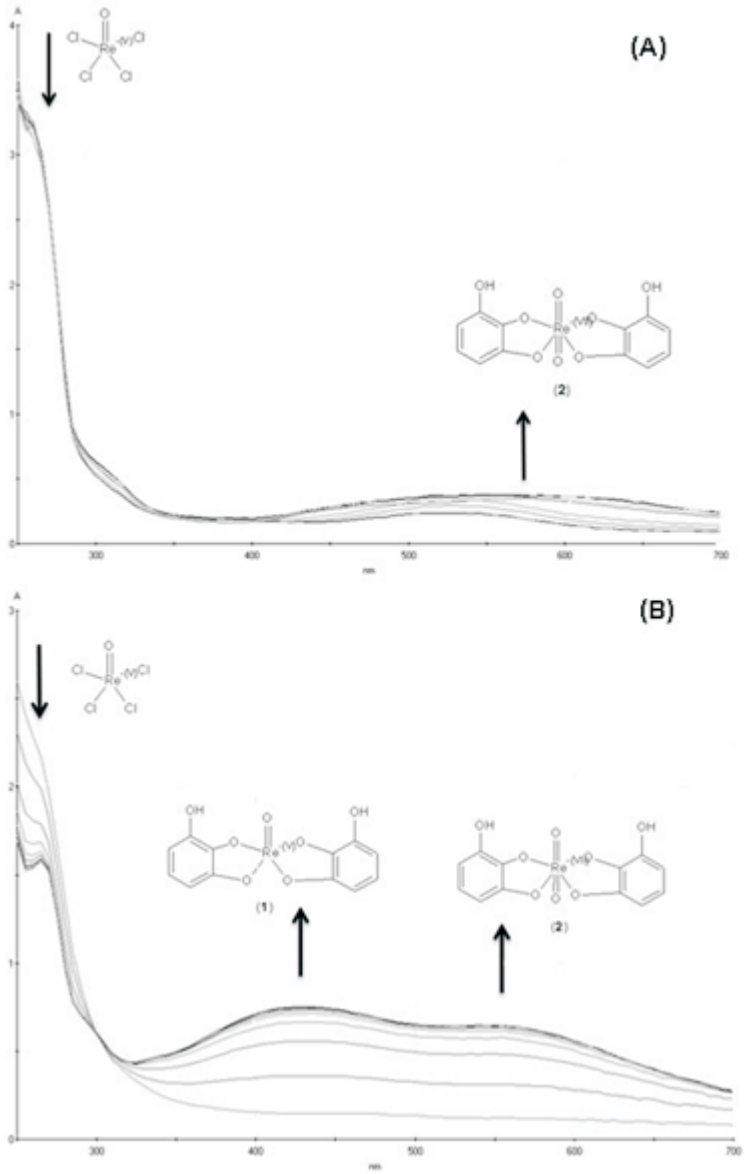


Fig. 3. UV/VIS absorption spectra of transition reaction between complexes (**1**) and (**2**) in deoxygenated ACN (A) and untreated ACN (B) monitored in time interval 60 min. Absorption maxima of complexes (**1**) and (**2**) and residual rhenium(+V) precursor are marked with arrows.

mixture. It is evident that reaction rate is the highest for 12 or 16 equivalents of triethylamine, the most significant change of the rate was observed in the interval of 48 equivalents of triethylamine.

4. Conclusions

Presented kinetic study documents the complexity of chemical reactions between Re(+V) precursor and pyrogallol as a strongly bound ligand. Besides its evident chemical matter of interest, our results might be useful in radiopharmaceutical research, namely for optimization of the composition of rhenium diagnostic and palliative treatment kits.

In this work we demonstrated that mass spectrometry with electrospray ionization is a useful tool both for structure characterization of rhenium complexes and for monitoring of their chemical transformations in real time. The mass spectra obtained with ESI gave unambiguous information about molecular weights and the structures of studied complexes, because intact molecular ions and structurally characteristic fragments were observed. Identified complex compound enabled to propose reaction scheme describing basic reaction pathways of studied complexes.

We also demonstrated the rate of complexes forming reactions for rhenium complexes and the depending of this rate on air access and on the addition of triethylmine. The results of ESI/MS kinetic studies were in agreement with independent UV/VIS absorption measurements and together show problematic behavior of rhenium complexes with oxygen donor ligands in non-aqueous solutions and conversion reactions between complexes. Without perfect knowledge of these transitions the drug application to patient's body will be dangerous.

Acknowledgments

This research was carried out within the framework of the project of the Specific University Research (SVV260205).

References

[1] Abrams M.J., Murrer B. A.: *Science* **261** (1993), 725–730.
[2] Gerloch M., Constable E. G.: *Transition Metal Chemistry.* Weinheim, VCH 1994.
[3] Colton R.: *The Chemistry of Rhenium and Technetium.* London, Interscience 1965.
[4] Wojcicki A., Basolo F.: *J. Am. Chem. Soc.* **83** (1961), 520–525.
[5] Angelici R. J., Basolo F.: *J. Am. Chem. Soc.* **84** (1962), 2495–2499.
[6] Angelici R. J., Basolo F.: *Inorg. Chem.* **2** (1963), 728–731.
[7] Hieber W., Fuchs H.: *Z. Anorg. Allgem. Chem.* **248** (1941), 256–268.
[8] Zingales F., Sartorelli U., Canziani F., Raveglia M.: *Inorg. Chem.* **6** (1966), 154–157.
[9] Casy Ch. P., Nash J.R., Yi Ch. S., Selmeczy A.D., Chung S., Powell D.R., Hayashi R.K.: *J. Am. Chem. Soc.* **120** (1997), 722–733.
[10] Wang Y., Espenson J.H.: *Inorg. Chem.* **41** (2002), 2266–2274.
[11] Dixon J., Espenson J.H.: *Inorg. Chem.* **41** (2002), 4727–4731.
[12] Han Y., Harlan C. J., Stoessel P., Frost B.J., Nornton J.R., Miller S., Bridgewater B., Xu. Q.: *Inorg. Chem.* **40** (2001), 2942–2952.

[13] Schibli R., Dumas C., Petrig J., Spadola L., Scapozza L., Garcia-Garayoa E., Schubiger P. A.: *Bioconjugate Chem.* **16** (2005), 105–112.
[14] McPherson L.D., Drees M., Khan S.I., Strassner T., Abu-Omar M.M.: *Inorg. Chem.* **43** (2004), 4036–4050.
[15] Lente G., Espenson J.H.: *Inorg. Chem.* **39** (2000), 4809–4814.
[16] Gable K.P:, Brown E.C.: *J. Am. Chem. Soc.* **125** (2003), 11018–11026.
[17] Vikuturi S., Chapman G., Ahmad I., Nicholas K.M.: *Inorg. Chem.* **49** (2010), 4744–4746.
[18] Owens G.S., Durazo A., Abu-Omar M.M.: *Chem. Eur. J.* **8** (2002), 3053–3059.
[19] Kojima R., Aika K.: *Appl. Cat. A* **209** (2001), 317–325.
[20] Holder G.N., Bottomley L.A.: *Inorg. Chim. Acta* **194** (1992), 133–137.
[21] Van Kirk Ch.C., Béreau V., Abu-Omar M.M., Evans D.H.: J. *Electroanal. Chem.* **541** (2003), 31–38.
[22] Koudelková M., Jedináková-Křížová V.: *J. Chrom. A.* **990** (2003), 317–323.
[23] Kohlíčková-Koudelková M., Jedináková-Křížová V. Deyl Z.: *Electrophoresis* **23** (2002), 245–248.
[24] Kohlíčková M., Jedináková-Křížová V., Konířová R.: *J. Radioanal. Nuc. Chem.* **242** (1999), 545–549.
[25] Nicholson T., Kramer D.J., Davison A., Jones A.G.: *Inorg. Chim. Acta* **353** (2001), 177–182.
[26] Valliant J.F., Riddoch R.W., Hughes D.W., Roe D.G., Farconnier T.K., Thornback J.R.: *Inorg. Chim. Acta* **352** (2001), 155–163.
[27] Henderson V., Nicholson B.K., McCaffrey L.J.: *Polyhedron* **17** (1998), 4291–4313
[28] Hori H., Ishitani O., Koike K., Takeuchi K.: *Anal. Sci.* **12** (1996), 587–590.
[29] Hori H., Ishihara J., Koike K., Takeuchi K., Ibusuki T., Ishitani O.: *Chem. Lett.* **3** (1997), 273–274.
[30] Tisato F., Bolzati C., Porchia M., Refosco F.: *Spectrom. Reviews* **23** (2004), 309–332.
[31] Wyatt M.F.: *J. Mass Spectrom.* **46** (2011), 712–719.
[32] Wyatt M.F., Stein B.K., Brenton A.G.: *Anal. Chem.* **78** (2006), 199–209.
[33] Van Berkel G.J.: *Europ. J. Mass Spectrom.* **9** (2003), 539–562.
[34] Petroselli G., Mandal M.K., Chen L.C., Ruiz G.T., Wolcan E., Hiraoka K., Nonami H., Erra-Balsells R.: *J. Mass Spectrom.* **47** (2012), 313–321.
[35] Day E.F., Payne T.A., Holt C.A.: *Rapid Commun. Mass Spectrom.* **21** (2007), 903–910.

New Polish reference material for the analysis of PAHs in the soil – determination of certified values and homogeneity

Anna Kiełbasa*, Bogusław Buszewski

Chair of Environmental Chemistry and Bioanalytics, Faculty of Chemistry, Nicolaus Copernicus University, 7 Gagarin St, 87-100 Toruń, Poland ✉ *kielbasa.am@gmail.com*

Keywords
accelerated solvent extraction
certified reference material
homogeneity
PAH
soil

Abstract
Accelerated solvent extraction with the use of a dichloromethane-acetone mixture (1:1 ratio) was applied to extraction of six PAH. This method allowed the recovery ranging from 70 to 115% and good repeatability (*SD* = 1.9–5.7). In order to determination method, liquid chromatography with the fluorescence detector and gas chromatography–mass spectrometry were applied. The validation of the method for PAH extraction and determination in soil was necessary for the purposes of further certification tests aimed at determination the certified value of the new reference material and its homogeneity.

1. Introduction

Reference materials (RM) and certified reference materials (CRM) are an indispensable part of systems that control and evaluate the quality of analytical results. Certified reference materials have to meet four basic criteria: analyte content determined with the required precision and accuracy, homogeneity, stability and cohesive documentation called certificate [1–3]. All the reference material guidelines covering terminology, production methods, certification, quality assurance, and application are contained in ISO guides no. 30 to 35 [3].

Homogeneity is determined for one batch of material, and the results have to be consistent with the values of the same parameters (with estimated uncertainty) for a different material batch. There are two types of homogeneity: within-bottle and between-bottle. Determination the certified value is done as a result of interlaboratory tests. All certified values are determinated using validated analytical methods [1, 4].

In the new Polish candidate for reference material, six compounds belonging to the PAH group were determined. These were pyrene, benzo[*a*]anthracene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, and indeno[1,2,3-*cd*]pyrene. With the use of the validated method, the PAH contents in the new candidate for reference material and homogeneity were determined.

2. Experimental

2.1 Reagents and chemicals

The certified standard of the six PAH (pyrene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]anthracene, and indeno[1,2,3-*cd*]pyrene) in acetonitrile was supplied by LGC Standards. The concentration of each compound was declared 10 ng/mL. For optimization analysis of the PAH in soil was supplied by CRM 141 and BAM U013-b. The candidate for the new reference materials was kept in closed glass (dark bottles). Each of them contained 50 grams of soil. These materials were stored at 4 °C. The materials were powdered and homogenized. Dichloromethane, acetone and acetonitrile were used. Water used was obtained from a MilliQ system. Nitrogen was of purity higher than 99%. The sample was filtered through syringe filters (PTFE, 13 mm diameter and 0.22 μm pore size).

2.2 Instrumentation

Accelerated solvent extraction (ASE 100, Dionex) was used for extraction. HPLC/FLD analysis was carried out by application of a liquid chromatograph (Agilent Technologies 1100 Series and 1260 Infinity). The chromatograph was performed on a non-polar column YMC PAH (250 mm × 3.0 mm; 5 μm). The analytes were determined by gradient elution with an acetonitrile–water binary system and subsequent fluorescence detection. 5 μL of the extract was injected. The column temperature was set at 30 °C. The mobile phase flowed at the rate of 1 mL/min. A gas chromatograph was fitted with a selective mass detector (Agilent Technologies 6890 N and 5975 Series) and a 30 m × 0.25 mm × 0.25 μm capillary column (ZB-5MS, Phenomenex). An aliquot (1μL) of the acetonitrile extract was injected. Helium was the carrier gas and flowed at a rate of 1.1 mL/min. A splitless injection mode was used in the analysis.

3. Results and discussion

From the basic standard solution, six working standard solutions were prepared by dilution to the concentrations of 100, 150, 200, 300, 400 and 500 μg/L. A chromatographic analysis was performed with the use of HPLC/FLD and GC/MS. For each of the compounds examined, an analytical curve as well as detection and determination limits were determined (Table 1). The detection and determination limits found are low which allows the determination of the PAH in a broad range of concentration values. It is possible to perform both, the qualitative as well as quantitative analysis. The analytical curves exhibited good linearity throughout the whole working range.

Table 1
Limit of detection and limit of quantification for six PAH.

Compound	HPLC/FLD		GC/MS	
	LOD [μg/L]	LOD [μg/L]	LOD [μg/L]	LOD [μg/L]
Pyrene	5.23	15.7	18.8	56.4
Benzo[*a*]anthracene	5.56	16.7	10.6	31.9
Benzo[*b*]fluoranthene	4.58	13.7	14.2	42.5
Benzo[*k*]fluoranthene	4.67	14.0		
Benzo[*a*]pyrene	5.49	16.5	25.6	76.8
Indeno[1,2,3-*cd*]pyrene	4.95	14.9	26.5	79.4

The 1.0 g sample (CRM 141, BAM U013-b and candidate for reference material) was carefully mixed with roast sand and placed in an extraction cell. The extraction ran at 100 °C in three static cycles, 5 min each. The extract was then concentrated in a mild stream of nitrogen at the temperature 30°C. The final volume of each sample was 1 mL and acetonitrile was the solvent. Each sample was filtered out with the use of Teflon syringe filters. Each samples was chromatographic analysis. CRM 141 and BAM U013-b were used for validation of the PAH in soil. Recovery and repeatability are shown in Table 2.

The PAH were extracted from the candidate for new certified reference materials for determination certified values. Results are shown in Table 3.

Table 2
Recovery and repeatability method analysis of PAH.

Compound	HPLC/FLD				GC/MS			
	CRM-141		BAM U013-b		CRM-141		BAM U013-b	
	Recovery [%]	SD	Recovery [%]	SD	Recovery [%]	SD	Recovery [%]	SD
Pyrene	93	4.1	106	2.9	86	3.7	92	3.2
Benzo[*a*]anthracene	85	4.9	99	3.0	115	2.2	87	4.8
Benzo[*b*]fluoranthene	97	4.5	87	4.1	107	5.7	106	4.9
Benzo[*k*]fluoranthene	96	4.8	87	3.5				
Benzo[*a*]pyrene	73	1.9	95	3.8	70	1.9	86	3.5
Indeno[1,2,3-*cd*]pyrene	97	3.2	105	3.5	114	4.1	95	4.4

Table 3
Concentration PAH in new certified reference material's candidate.

Compound	HPLC/FLD		GC/MS	
	Concentration [µg/kg]	SD	Concentration [µg/kg]	SD
Pyrene	4638	162	4765	94
Benzo[*a*]anthracene	533	37	487	42
Benzo[*b*]fluoranthene	625	39	1016	34
Benzo[*k*] fluoranthene	310	28		
Benzo[*a*]pyrene	545	48	499	46
Indeno[1,2,3-*cd*]pyrene	1557	64	561	51

By HPLC/FLD, it is possible to quantitatively determine five compounds (pyrene, benzo[*a*]anthracene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene) in the new reference material. Due to interferants which elute with indeno[1,2,3-*cd*]pyrene, it is not possible to analyse it qualitatively with liquid chromatographic methods. GC/MS was applied in the analysis of this compound in soil (Fig.1).

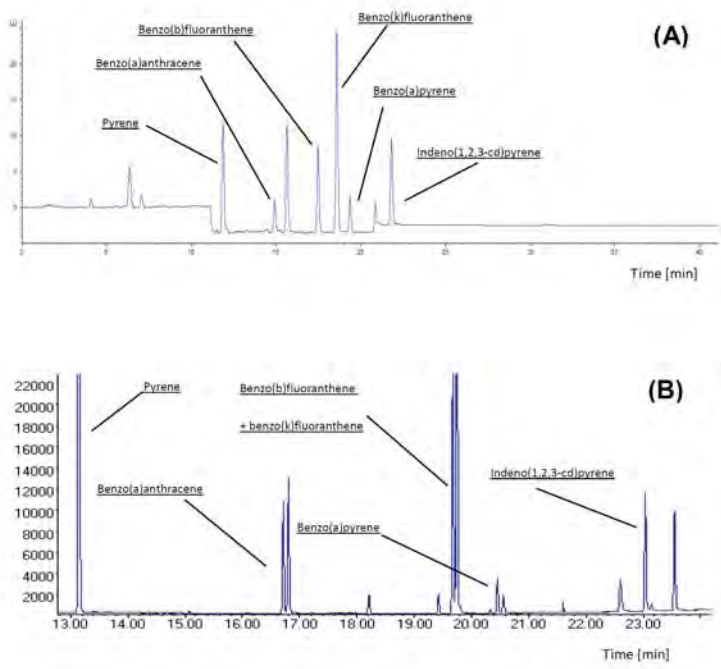


Fig. 1. Chromatograms of PAHs in new certified reference material by (A) HPLC/FID analysis, and (B) GC/MS analysis.

Table 4
Coefficients of variation for analysis, within-bottle and between-bottle homogeneity.

Compound	CV analysis %	CV WB %	CV BB %
Pyrene	1.9	10	3.7
Benzo[*a*]anthracene	2.7	19	5.4
Benzo[*b*]fluoranthene	3.4	17	5.3
Benzo[*k*] fluoranthene	6.7	25	4.4
Benzo[*a*]pyrene	5.2	24	5.2
Indeno[1,2,3-*cd*]pyrene	8.0	20	5.7

For homogeneity PAH were extracted several times from few randomly selected bottles. Variation coefficients were calculated for within-bottle homogeneity and between-bottle homogeneity (Table 4).

4. Conclusions

The accelerated solvent extraction method with the use of the dichloromethane-acetone mixture is quick method characterized by the highest recovery and, at the same time, repeatability. Preparing the sample for the analysis with the use of HPLC/FLD and GC/MS is simultaneous. It is cost-efficient due to smaller amounts of the solvent needed for the analysis and shorter total procedure time.

A candidate for the new reference material fulfills two criteria required for certification process. Certification values were determined with sufficient precision and accuracy. For this purpose was validated analytical method. The determined coefficients of variation indicate high within-bottle and between-bottle homogeneity.

The material will be very useful in laboratory tests when examining soil collected from areas affected by ecocatastrophes or struggling with the environmental contamination with the PAH.

Acknowledgments

This project was financed in the framework of grant entitled: "Production and attestation of new types of reference materials crucial for achieving European accreditation for polish industrial laboratories (MODAS)" attributed by the National Center for Research and Development, no. INNOTECH-K1/IN1/43/158947/NCBR/12.

References

[1] Namieśnik J., Zygmunt B.: *Sci. Total Environ.* **228** (1999), 243–257.
[2] Konieczka P.: *Crit. Rev. Anal. Chem.* **37** (2007), 173–190.
[3] International Organization for Standarization (Geneva, Switzerland): Quality system gudelines for the production of reference materials, ISO/IEC Guide 34, 1996; Terms and definitions used in connection with reference materials, ISO Guide 30, 1992; Contents of certificates of reference materials, ISO Guide 31, 1981; Certification of reference materials: general and statistical principles, ISO/IEC Guide 35, 1989; Calibration in analytical chemistry and use of certified reference materials, ISO Guide 32, 1997; Uses of Certified Reference Materials, Revised ISO Guide 33, 2000.
[4] Linsinger T.P.J., Pauwels J., van der Veen A.M.H., Schimmel H., Lamberty A.: *Accred. Qual. Assur.* **6** (2001), 20–25.

Influence of electrode preparation on electrocatalytic activity of water soluble porphyrazine complex to hydrogen

Monika Klusáčková[a, b, *], Pavel Janda[a], Hana Tarábková[a]

[a] *J. Heyrovský Institute of Physical Chemistry of ASCR, v.v.i., Dolejškova 3, 182 23 Prague 8, Czech Republic* ✉ *monika.klusackova@jh-inst.cas.cz*
[b] *Department of Analytical Chemistry, Faculty of Science, Charles University in Prague, Hlavova 8, 128 43 Prague 2, Czech Republic*

Keywords
atomic force microscopy
backscattering microscopy
cyclic voltammetry
electrocatalysis
water soluble phthalocyanine

Abstract
The influence of electrode preparation and used electrode substrate on electrocatalytic activity of *N,N´,N´´,N´´´*-tetramethyl-tetra-3,4-pyridinoporphyrazinocobalt (CoTmt-3,4-ppa) complex deposited on electrode surface for hydrogen reaction has been studied. Electrochemical deposition, spontaneous adsorption, and spin coating have been used to the modification of two different electrode substrates: annealed gold and highly oriented pyrolytic graphite. Prepared electrodes have been characterized using cyclic voltammetry, backscattering VIS spectroscopy and atomic force microscopy. The detailed comparison of surface morphology, surface coverage, surface roughness parameters, and thickness of deposited layers of CoTmt-3,4-ppa mediator on hydrogen reaction has been investigated.

1. Introduction

Porphyrazines and phthalocyanines, synthetic analogues of porphyrins, are structurally related macrocyclic compounds with two-dimensional 18 π-electron conjugated system, in which can be incorporated more than 70 different metal or non-metal elements. These complexes are ideal components in variety applications because of their synthetic versatility and easy modification of the benzene ring. Water-soluble compounds exist as freely associated aggregates that are not chemically bound in aqueous solution [1]. Although the aggregation tendency is problematic, solubility in water is an advantage for many applications. The advantage of complex *N,N´,N´´,N´´´*-tetramethyl-tetra-3,4-pyridinoporphyrazinocobalt (CoTmt-3,4-ppa) reported in this work is a lower tendency to form aggregates in solution compared to other water-soluble phthalocyanine.

Porphyrazine and phthalocyanine compounds have been reported to provide electrocatalytic activity towards a considerable number of analytes [2–6] and adsorbed onto electrodes show better catalytic activity than when are used as

homogeneous catalysts. The key factor that allows them to serve as mediators in many electron transfer reactions is their reversible redox chemistry, high thermal and chemical stability [7, 8]. They are able to form insoluble, thin layer due to strong intermolecular interaction on different types of electrode substrates with significant electrocatalytic properties.

Important aspects to prepare films for gas sensors are morphology and thickness of active layers [9]. Thin layers phthalocyanine derivatives on electrode surface increase the surface area and can serve as sensitive gas sensors. This can be achieved in various ways such as electrochemical deposition, by drop casting, and spin coating of phthalocyanine solution on the electrode substrate. Thickness and morphology of deposited films can be controlled in these methods by amount of phthalocyanine and deposition time.

Hydrogen catalysis is a significant scientific field since hydrogen evolution and hydrogen oxidation are importance in developing and improving in electrochemical devices such as fuel cells [10] and water electrolysers [11]. Possible hydrogen catalyst candidates include various pure metals such as platinum, palladium, ruthenium, and theirs alloys. These metals are resource-limited, expensive, and irreversibly inactivated by common trace impurities. One way to resolve this problem is by modifying conventional electrodes with catalytic active complexes such as metallophthalocyanines.

The presented work reports how the method of deposition and used substrate affect electrocatalytic activity of water-soluble porphyrazine mediator. The electrocatalytic activity of CoTmt-3,4-ppa complex to hydrogen in aqueous solution at laboratory temperature was investigated.

2. Experimental

2.1 Reagents and chemicals

The water-soluble powder Co(II)Tmtppa$(CH_3SO_4)^{4-}$ was synthesized and purified by the group of Professor A. B. P. Lever from York University, Toronto, Canada [2]. Stock solutions of the CoTmt-3,4-ppa with a concentration of 1×10^{-3} mol dm^{-3} was prepared in phosphate buffer (pH = 4.3) and stored in cold and dark. Phosphate buffer serving as supporting electrolyte was prepared by mixing of 0.1 mol dm^{-3} solutions of NaH_2PO_4 and Na_2HPO_4 (Merck, Germany) in distilled water. Phosphoric acid and sodium hydroxide (all p.a., Lachema, Czech Republic), 0.1 mol dm^{-3} each, were used for pH adjustments. All chemicals used for buffer preparation were analytical grade and used without further purification. For all the measurements was used deionized water (Milli-Q system Gradient, Millipore, resistivity 18.2 MΩ cm). The gases used in this work include argon 6.0 and hydrogen 6.0 (all 99.9999% purity, Messer, Czech Republic).

2.2 Instrumentation

Cyclic voltammetry (CV) experiments were performed using a three-electrode system controlled by the potentiostat/galvanostat Wenking POS 2 (Bank Elektronik, Germany) with CPC-DA software (Bank Elektronik, Germany). Electrode materials such as gold substrate Au(111)-coated glass slides (Gold arrandee™/Au(111) Germany) with a thickness of gold film 250 ± 50 nm in the size of the glass slide 12 × 12 × +/– 0.2 mm and highly oriented pyrolytic graphite (HOPG) 12 × 12 × 2 mm (HOPG ZYB Grade, Structure Probe Inc., USA) were used as working electrode. Saturated calomel electrode (SCE) and platinum wire served as reference and auxiliary electrode, respectively. The gold-coated glass electrode was cleaned by flame annealed in a Bunsen burner to obtain Au(111) terraces. This procedure was repeated three times after air cooling for a short time. The HOPG electrode was cleaned using adhesive tape Scotch by removing several layers of the surface. All electrochemical measurements were carried out in 0.1 mol dm^{-3} phosphate buffer and deoxygenated by argon at room temperature.

The fibre optic spectroscopic instrumentation consisted of spectrometer SD 1000 (Ocean Optics, New Zealand) with optical fibre (Ocean Optics, USA). The halogen lamp Fiber-Lite PL-800 (Dolan-Jenner, USA) was used as the source of irradiation in the range of 400–800 nm. The spectra were measured in the back-scattering mode from the electrode/solution interface and were recorded by the OOI Operating Base 1.52 (Ocean Optics, New Zealand) software package. All experiments were performed in the solution deoxygenated by argon 6.0 at room temperature.

The surface topography of electrode substrates was examined by ex situ atomic force microscopy Multimode Nanoscope IIIa (Veeco, USA) in utilizing tapping mode with silicon tip (OTESPA, 42 Nm-1, 300 kHz, Veeco, USA). The atomic force microscopy (AFM) topography was analysed by commercial Nanoscope III Software version5.12r5 (Veeco, USA) and the determination of the surface roughness parameters was performed by using Gwyddion software version 2.41 (Czech Metrology Institute, Brno, Czech Republic).

2.3 Preparation of modified electrode

Electrochemical deposition of CoTmt-3,4-ppa on the electrode substrate was performed by recording successive cyclic voltammetric scans in a potential range from –0.8 to 0.1 V versus SCE in a pH = 4.3 buffer solution containing 1×10^{-5} mol dm^{-3} CoTmt-3,4-ppa. The number of cycle was adjusted to obtain desired thickness of deposited layers. Adsorption of mediator on the electrode was realized by 60 min drop casting of 1×10^{-3} mol dm^{-3} CoTmt-3,4-ppa. Excess solution was removed from the surface. Spin-coating of porphyrazine was formed by spin coating (500 rpm) of 1×10^{-3} mol dm^{-3} CoTmt-3,4-ppa solution. Excess

solution is spun off the surface. After completion of deposition techniques, the modified electrodes (labelled CoTmt-3,4-ppa/HOPG and CoTmt-3,4-ppa/Au) were further characterized in the buffer solution in the absence of CoTmt-3,4-ppa.

3. Results and discussion

Firstly, modified electrodes (CoTmt-3,4-ppa/HOPG and CoTmt-3,4-ppa/Au) prepared by three different methods were characterized by cyclic voltammetry in the buffer solution. Electrochemical behaviour and surface coverage of CoTmt-3,4-ppa on electrode were studied. Electrochemical results were completed by ex situ AFM to have better understanding of deposition processes. Profile analysis and AFM images showed smoother nanomorphology of CoTmt-3,4-ppa deposited on HOPG surface compare to Au substrate. It was found out that surface roughness parameters and thickness of deposited layers were influenced the used substrate and the type of deposition method.

Further, VIS absorption spectroscopy in the backscattering mode allow monitored processes of the spontaneous adsorption and the electrochemical deposition of the complex CoTmt-3,4-ppa on electrode substrates. The process of deposition resulted in the spectral change consisted of the decrease in the sharp Q-band at 670 nm and the simultaneous formation of a new broad absorption band centred at 472 nm. This process corresponded to reduction of the central metal and the formation of active layer on the electrode surface. The stability of films was monitored by electrochemical and spectral change in the buffer solution in the absence of CoTmt-3,4-ppa.

Finally, cyclic voltammetry and potentiometry were used to investigate the possible practical application of porphyrazine as electrocatalysts for hydrogen. The electrocatalytic activity of the mediator coated on electrode surface was tested in a phosphate buffer aqueous solution at different pHs. It was detected that the electrocatalytic activity of the complex varies with the changing pH due to different mechanism between hydrogen and CoTmt-3,4-ppa. Further, it was found that the type of substrate and the method of preparing the modified electrode also affected the electrocatalytic activity of complex. The potential-time response of CoTmt-3,4-ppa thin layer to hydrogen makes it possible to development brand-new potentiometric sensor.

4. Conclusions

Cobalt porphyrazine thin films were deposited on annealed gold and highly oriented pyrolytic graphite substrates by adsorption, spin-coating and electrochemical deposition. After that, thin films were characterized electrochemical, spectroelectrochemical, and microscopically methods. The detailed comparison of AFM results with electrochemical and spectrochemical measurements made it possible to gain further insight in the processes of thin layer

during the modification procedure and electrocatalytic behaviour. It was observed that electrochemical behaviour and electrocatalytic activity of CoTmt-3,4-ppa complex to hydrogen were significantly affected by electrode modification technique and material of electrode substrate.

Acknowledgments

This work was financially supported by grant project SVV260205 of Charles University of Prague and the Grant Agency of the Czech Republic (Czech Science Foundation) contract No. 14-14696S.

References

[1] Biyiklioğlu Z.: *Synthetic Metals* **162** (2012), 26–34.
[2] Tse Y.H., Janda P., Lever A.B.P.: *Anal. Chem.* **66** (1994), 384–390.
[3] Thamae M., Nyokong T.: *J. Porphyrins Phthalocyanines.* **5** (2001), 839–845.
[4] Zhang J., Tse Y.H., Pietro W.J., Lever A.B.P.: *J. Electroanal. Chem.* **406** (1996), 203–211.
[5] Zhang J., Pietro W.J., Lever A.B.P.: *J. Electroanal. Chem.* **403** (1996), 93–100.
[6] Thamae M., Nyokong T.: *J. Electroanal. Chem.* **470** (1999), 126–135.
[7] Chen J., Zhang J., Tse Y.H., Janda P., Christendat D., Lever A.B.P.: *J. Porphyrins Phthalocyanines.* **10** (2006), 1238–1248.
[8] Barrera C., Zhukov I., Villagra E., Bedioui F., Páez M.A., Costamagna J., Zagal J.H.: *J. Electroanal. Chem.* **589** (2006), 212–218.
[9] Jafari M.J., Azim-Araghi M.E., Barhemat S., Riyazi S.: *Surf. Interface Anal.* **44** (2012), 601–608.
[10] Varcoe J.R., Slade R.C.T., Wright G.L., Chen Y.: *J. Phys. Chem. B.* **110** (2006), 21041–21049.
[11] Nguyen M.T.D., Ranjbari A., Catala L., Brisset F., Millet P.: *Coord. Chem. Rev.* **256** (2012), 2435–2444.

Chiroptical spectroscopy as a sensitive tool for the conformational analysis of antimicrobial peptides

Lucie Kocourková[a,*], Pavlína Novotná[a], Sabína Čujová[b], Václav Čeřovský[b], Marie Urbanová[c], Vladimír Setnička[a]

[a] *University of Chemistry and Technology, Prague, Faculty of Chemical Engineering, Department of Analytical Chemistry, Technická 5, 166 28, Prague 6, Czech Republic* ✉ *lucie.kocourkova@vscht.cz*
[b] *The Czech Academy of Sciences, Institute of Organic Chemistry and Biochemistry, Flemingovo náměstí 2, 166 10, Prague 6, Czech Republic*
[c] *University of Chemistry and Technology, Prague, Faculty of Chemical Engineering, Department of Physics and Measurements, Technická 5, 166 28, Prague 6, Czech Republic*

Keywords
antimicrobial peptides
circular dichroism
conformation
liposomes
model membranes

Abstract
We tested the utilization of electronic and vibrational circular dichroism for the conformational study of antimicrobial peptides on two examples: melectin and antapin in different solutions of liposomes and micelles mimicking bacterial or eukaryotic membranes. The results show significant content of the α-helical conformation in an environment mimicking the bacterial membrane, while it was considerably low in the eukaryotic membrane-mimicking environment.

1. Introduction

Chiroptical methods, as vibrational (VCD) and electronic circular dichroism (ECD) are based on the principle of a difference in absorption of the left- and right-circularly polarized light by chiral molecules [1]. They are therefore very useful for the studies of a 3D structure from small molecules to biopolymers. Both the methods characterize conformations by differing spectral patterns. Thus, we tested their potential for conformational studies of antimicrobial peptides in model membranes, because the level of antimicrobial and hemolytic activities of cationic α-helical antimicrobial peptides depends on their cationicity, hydrophobicity, and also on their propensity to adopt an amphipathic α-helical conformation on the cell membrane surface. While, the ECD spectroscopy has long been established as one of the main spectroscopic techniques to study antimicrobial peptides, the application of the VCD spectroscopy is quite new for such studies, especially in the systems with membrane-mimicking environments.

Hence, we studied melectin and antapin antimicrobial peptides originally isolated from different bees [2, 3] in an environment mimicking bacterial or

eukaryotic membranes. As models of such membranes various liposomes and micelles were used. Methods of circular dichroism were also supplemented by infrared (IR) spectroscopy.

2. Experimental

2.1 Preparation of peptide samples, micelles and liposomes

The antimicrobial peptides melectin (GFLSILKKVLPKVMAHMK-NH_2) and antapin (GLLSALRKMIPHILSHIKK-NH_2) were synthesized, purified and fully characterized in our laboratory [2, 3]. Before the measurements, the peptides were dissolved in a 0.1 mol L^{-1} HCl solution and then lyophilized to remove TFA. Peptides modified in this way were dissolved in liposomal solutions, deuterium oxide (D_2O) or 40% solution of deuterated trifluoroethanol (TFE-d_3) in deuterium oxide to obtain the solution of a concentration 50 g L^{-1}. The samples with sodium dodecyl sulphate (SDS) micelles were prepared by dissolving the peptide in a phosphate buffer (10 mmol L^{-1}, pH = 7.5) and then the stock solution of the SDS micelles (concentration of 160 g L^{-1} in the phosphate buffer, 10 mmol L^{-1}, pH = 7.5) was added to obtain the solution of a peptide concentration 50 g L^{-1}.

The 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dimyristoyl--*sn*-glycero-3-phospho-(1'-rac-glycerol) sodium salt (DMPG) lipids were used for the preparation of liposomes according to the standard procedures described in the literature[4, 5]. The concentration of the lipids in the resulting solution was 100 g L^{-1}. The dynamic light scattering method confirmed quite narrow size distribution of the liposomes with a maximum at 123±2 nm.

2.2 Electronic and vibrational circular dichroism and Fourier transform infrared spectroscopy

The VCD and IR absorption spectra were measured on a Fourier transform IR (FT-IR) IFS 66/S spectrometer[6] and the ECD spectra on a J-815 spectrometer. The samples were measured with standard conditions and settings for peptides, described previously [7, 8].

The spectra were corrected for a baseline by subtracting the spectra of liposomes-free or micelles-free solution measured under the same experimental conditions. The final spectra were expressed as a molar circular dichroism $\Delta\varepsilon$ (L $mol^{-1}cm^{-1}$) per residue or a molar absorption coefficient ε (L $mol^{-1}cm^{-1}$) per residue. The ECD spectra were analyzed in the spectral range 190–240 nm with the help of a Dichroweb [9, 10] application using CDSSTR [11] method and Dataset 4.

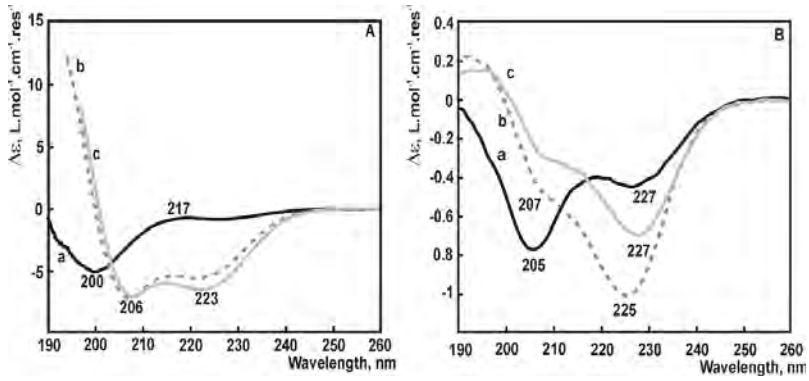


Fig. 1.
(A) The ECD spectra of the melectin peptide in deuterium oxide (a), in the 40% solution of TFE-d_3 (b) and in the solution of the SDS micelles (c).
(B) The ECD spectra of the antapin peptide in deuterium oxide (a), in the 40% solution of TFE-d_3 (b) and in the solution of the SDS micelles (c).

3. Results and discussion

In deuterium oxide, the antapin and melectin peptides predominantly assumed the polyprolin II (PPII) type conformation [12] as it was observed in the ECD and VCD spectra of both peptides (Fig. 1 and 2). The ECD spectra show, besides the characteristic bands of the PPII type conformation, a negative band at ~227 nm indicating a low content of the α-helical conformation which was also confirmed by the ECD spectral analysis using the Dichroweb application: a 10 and 4% content of this conformation was found for melectin and antapin, respectively (Table 1).

Table 1
The estimated percentual content of the individual conformations in the structures of melectin and antapin in different environments determined from the ECD spectra by using the Dichroweb application.

Evironment	Melectin				Antapin			
	α-Helix	Strand	Turn	Unordered	α-Helix	Strand	Turn	Unordered
Deuterium oxide	10	30	24	36	4	45	19	31
40% TFE-d_3	69	9	8	13	7	35	24	33
SDS	63	13	11	12	8	34	25	33
DOPC/DMPG (2:1)	53	15	12	19	19	26	27	27
DOPC	11	20	24	35	8	35	25	31

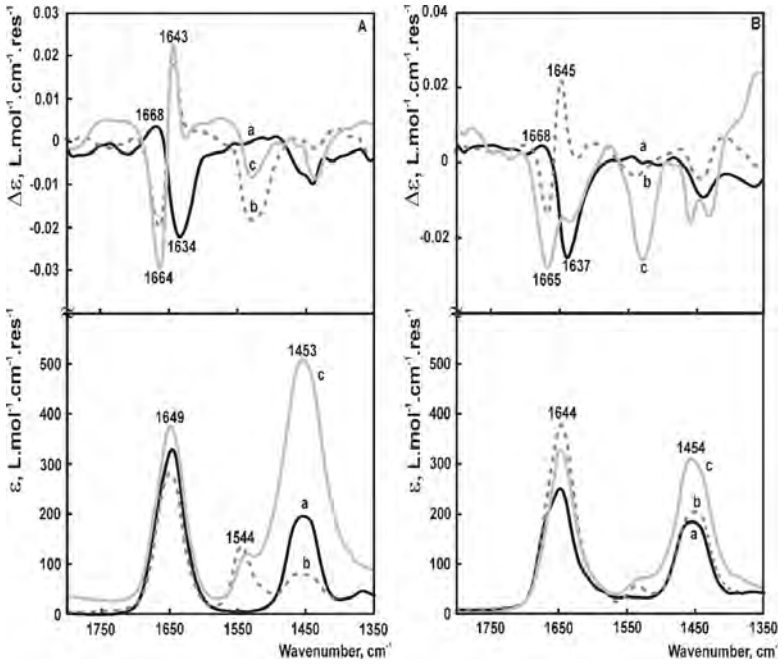


Fig. 2.
(A) The VCD (top) and IR (bottom) spectra of the melectin peptide in deuterium oxide (a), in the 40% solution of TFE-d_3 (b) and in the solution of the SDS of micelles (c).
(B) The VCD (top) and IR (bottom) spectra of the antapin peptide in deuterium oxide (a), in the 40% solution of TFE-d_3 (b) and in the solution of the SDS of micelles (c).

In the IR and VCD spectra (Fig. 2), our attention was focussed on the amide I´ region (~1670–1620 cm^{-1}), corresponding to the stretching vibration of the C=O group in the peptide bond [13]. In the VCD spectra in deuterium oxide, a negative couplet typical of the PPII type conformation[12] was observed for both melectin and antapin which confirmed our results from the ECD spectra.

A negative band at ~222 nm as a marker of the α-helical conformation[1] was observed in the ECD spectra (Fig. 1) of both the peptides in the solution of 40% TFE-d_3 and in the SDS micelles. The Dichroweb analysis of the data for melectin also revealed high content of the α-helical conformation (Table 1) which was in the TFE-d_3 solution caused by the ability of TFE-d_3 to promote the α-helical conformation[14]. For the antapin peptide, the estimated content of secondary structures was very similar with the content in deuterium oxide (Table 1).

The VCD spectra, where a positive couplet was observed for both the studied peptides in TFE-d_3 solution (Fig. 2), also indicated increased content of the α-helices in this environment. In the antapin solution of the SDS micelles, the observed "W" shape spectral pattern [15] indicates a mixture of the α-helical and PPII type conformations.

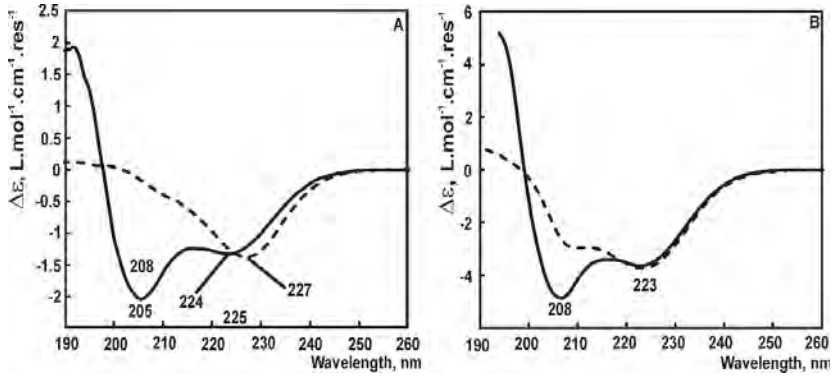


Fig. 3. The ECD spectra of the studied antimicrobial peptides (melectin – solid lines; antapin – dotted lines) in different environments: (A) DOPC, (B) DOPC/DMPG 2:1.

These results suggest that the melectin peptide has significantly higher propensity to adopt the α-helical conformation than the antapin peptide. Their interaction with membranes depends mostly on two peptide's parameters [16]: cationicity and hydrophobicity. As both peptides have similar positive charge, the difference may lie in their hydrophobicities (melectin –0.011 and antapin –0.077 according to the Eisenberg et al. theory [17]) as peptides with higher hydrophobicity usually adopt a conformation with higher fraction of the α-helices [18].

As a model of the bacterial membrane, liposomes composed of the mixture of DOPC and DMPG lipids in a molar ratio 2:1 were used [19]. The ECD spectra (Fig. 3) of both studied peptides reflect higher content of the α-helical fraction than in deuterium oxide, especially for the melectin peptide (~53%, Table 1). This is probably the cause of its observed higher antimicrobial activity [3, 4] than what was observed for antapin having only ~19% of the α-helices. The VCD spectra (Fig. 4) had the "W" shape spectral pattern indicating the presence of both α-helical and PPII type conformations, but in this case with a predominance of the α-helical conformation. This observation supports the ECD results.

Conversely, in the environment mimicking the eukaryotic membrane, as which the liposomes composed of the DOPC lipid were used, a lower content of the α-helical conformation was observed by both techniques (Fig. 3 and 4, from ECD: 11 and 8% for the melectin and antapin peptides, respectively). This correlates with desirable low hemolytic activity of both peptides.

4. Conclusions

By using VCD and ECD spectroscopy, we carried out a conformational study of the melectin and antapin peptides, which show quite high antimicrobial activity and relatively low hemolytic activity in various environments. As the level of these activities depends also on the peptide's propensity to adopt an amphipathic α-helical conformation on the cell membranes, we focussed mainly on the content

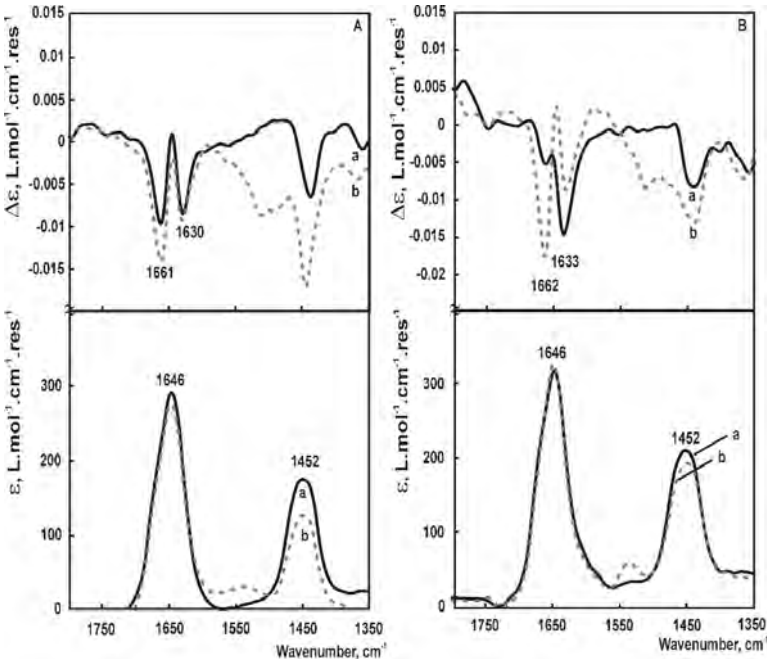


Fig. 4.
(A) The VCD (top) and IR (bottom) spectra of the melectin peptide in the solution of DOPC (a), DOPC/DMPG 2:1 (b) liposomes.
(B) The VCD (top) and and IR (bottom) spectra of the antapin peptide in the solution of DOPC (a), DOPC/DMPG 2:1 (b) liposomes.

of the α-helices in the peptides studied in the membrane-mimicking environments. The results from the ECD and VCD spectroscopies show its significantly higher content for both melectin and antapin in the environments mimicking the bacterial than the eukaryotic membranes which correlates with peptides' biological activities. Both methods, especially the not so common VCD technique, proved their effectiveness in the determination of the antimicrobial peptides conformation providing similar results.

Acknowledgments

The work was supported by the Czech Science Foundation (No. P208/11/0105). Authors also thank "Operational Program Prague – Competitiveness" (CZ.2.16/3.1.00/22197) and "National Program of Sustainability" (NPU) MSMT – LO1215; 34870/2013. Financial support from Specific University Research MSMT No 20/2015 (A1_FCHI_2015_003) is gratefully acknowledged.

References

[1] Berova N., Polavarapu P.L., Nakanishi K., Woody R.W.: *Comprehensive Chiroptical Spectroscopy, Applications in Stereochemical Analysis of Synthetic Compounds, Natural Products and Biomolecules.* New Jersey, Wiley 2012.
[2] Čeřovský V., Hovorka O., Cvačka J., Voburka Z., Bednárová L., Borovičková L., Slaninová J., Fučík V.: *Chembiochem* **9** (2008), 2815–2821.

[3] Čujová S., Veverka V., Buděšínský M., Bednárová L., Čeřovský V.: *J. Pept. Sci.* **20** (2014), S263–S264.
[4] Torchilin V., Weissig V.: *Liposomes.* New York, Oxford University Press 2003.
[5] Novotná P., Urbanová M.: *Anal. Biochem.* **427** (2012), 211–218.
[6] Urbanová M., Setnička V., Volka K.: *Chirality* **12** (2000), 199–203.
[7] Kykal S., Čeřovský V., Setnička V.: *Chem. Listy* **107** (2013), 250–254.
[8] Činčalová K., Čeřovský V., Hrubý T., Setnička V.: *Chem. Listy* **107** (2013), S229–S232.
[9] Whitmore L., Wallace B.A.: *Nucleic Acids Res.* **32** (2004), W668–W673.
[10] Sreerama N., Woody R.W.: *Anal. Biochem.* **287** (2000), 252–260.
[11] Johnson W.C.: *Proteins: Struct., Funct., Genet.* **35** (1999), 307–312.
[12] Berova N., Polavarapu P.L., Nakanishi K., Woody R.W.: *Comprehensive Chiroptical Spectroscopy, Instrumetation, Methodologies,* and *Theoretical Simulations.* New Jersey, Wiley 2012.
[13] Keiderling T.A.: *Curr. Opin. Chem. Biol.* **6** (2002), 682–688.
[14] Luo P., Baldwin L.R.: *Biochemistry* **36** (1997), 8413–8421.
[15] Yoder G., Pančoška P., Keiderling T.A.: *Biochemistry* **36** (1997), 15123–15133.
[16] Bahar A., Ren D.: *Pharmaceuticals* **6** (2013), 1543–1575.
[17] Eisenberg D., Schwarz E., Komaromy M., Wall R.: *J. Mol. Biol.* **179** (1984), 125–142.
[18] Blondelle S.E., Lohner K., Aguilar M.I.: *Biochim. Biophys. Acta Biomembr.* **1462** (1999), 89–108.
[19] Čujová S., Slaninová J., Monincová L., Fučík V., Bednárová L., Stokrová J., Hovorka O., Voburka Z., Straka J., Čeřovský V.: *Amino Acids* **45** (2013), 143–157.

Capillary electrophoretic study of solvent effects on complexation of dibenzo-18-crown-6 with potassium ions

Renáta Konášová[a, b, *], Jana Jaklová Dytrtová[b], Václav Kašička[b]

[a] *Department of Analytical Chemistry, Faculty of Science, Charles University in Prague, Hlavova 8, 128 43 Prague 2, Czech Republic*
[b] *Institute of Organic Chemistry and Biochemistry, The Czech Academy of Sciences, v.v.i., Flemingovo nám. 2, 166 10 Prague 6, Czech Republic* ✉ *konasova@uochb.cas.cz*

Keywords
affinity capillary electrophoresis
ethanol
methanol
non-covalent interactions
propan-2-ol

Abstract
Affinity capillary electrophoresis was used for investigation of solvent effect on stability constant of model complex of dibenzo-18-crown-6 with potassium ion in mixed hydro-organic solvents at different water/methanol, water/ethanol and water/propan-2-ol ratios. The apparent stability constant was obtained by non-linear regression analysis of the dependency of effective electrophoretic mobility of the dibenzo-18-crown-6 on potassium ion concentration in the background electrolyte. Prior to the regression analysis, the measured effective electrophoretic mobilities of the dibenzo-18-crown-6 were recalculated to the constant ionic strength. The obtained apparent stability constant of the complex was found to be strongly decreasing with increasing molar fraction of water in mixed solvents.

1. Introduction

Complex forming interactions of organic compounds with metal ions play very important role in organic catalysis, biochemistry and related fields like clinical diagnosis. Except these applications, an ability of organic compounds to bind metal ions is often used for extractions or in analytical chemistry when they are advantageously utilized for separation and/or determination of the various types of compounds. Knowledge of the basic characteristics of the complexes (stoichiometry and stability constant) is relevant for optimization of the reaction/separation conditions. However, the stability constants are much more frequently determined in pure solvents than in mixed solvents [1]. On the other hand, it is possible to follow the trend of changes of the stability constants in different solvent mixtures and to estimate the stability constant value in the particular composition of the mixed solvent.

The stability constants (K_{st}), of the complexes can be determined by various methods. The often used are calorimetry, chromatography, electromigration methods, spectrophotometry, MS, NMR and voltammetry [2, 3]. The suitability of

the methods for the stability constant determination mainly depends on stoichiometry and kinetic of the complexation and on solubility, accessibility and purity of the interacting compounds.

The main advantage of the electromigration methods is the small sample and electrolyte consumption and simplicity of the data evaluation [4, 5]. Moreover, the use of the sensitive detection technique (mass spectrometry, fluorescence detection) allows the determination of stability constants of strong complexes and complexes of slightly soluble compounds.

The aim of this study was to apply affinity capillary electrophoresis for investigation of influence of composition of mixed hydro-organic water/methanol, water/ethanol and water/propan-2-ol solvents on the stability constant of dibenzo-18-crown-6 complex with potassium ion.

2. Experimental

Dibenzo-18-crown-6 (98 %), methanol (HPLC grade), dimethyl sulfoxide (≥ 99.5 %), potassium chloride (analytical grade) and acetic acid (≥ 99.8 %) were purchased from Sigma-Aldrich, ethanol (absolute) and propan-2-ol were purchased from Penta (Czech Republic).

Affinity capillary electrophoresis experiments were performed in Agilent 7100 CE System (Agilent). The internally uncoated fused silica capillary (375/50 μm, OD/ID, 40/29 cm total/effective length) was used and UV-absorption detection at 206 nm was employed. The separation voltage varied from 6 kV to 26 kV. Background electrolyte consisted of 25 mM lithium hydroxide and acetic acid in concentration necessary for constant pH = 5.5. Portion of methanol (ethanol, propan-2-ol) in the solvent mixture varied from 0 to 100 % v/v. The pH was calculated using the pK_a values of acetic acid in the particular mixed solvents [6–8] and measured by pH meter CyberScan PC 5500 equipped with glass electrode. Concentration of potassium chloride in the background electrolyte varied from 0 to 10 mM (in the case of very weak complexes from 0 to 30 mM). Mixtures of the analyte (6×10^{-5} M) and DMSO (0.3 % v/v) in the background electrolyte were hydrodynamically introduced into the capillary by the pneumatically induced pressure (12 mbar) for 3 s. For the data evaluation and stability constant calculation, the programs MS Excel 2010 and Origin 8.5.1 were used.

The effective electrophoretic mobility of the ligand ($\mu_{eff,\,L}$) was calculated from migration time of the ligand ($t_{mig,\,L}$) and marker of electroosmosis ($t_{mig,\,EOF}$) using the equation

$$\mu_{\mathrm{eff,L}} = \frac{l_{\mathrm{tot}} l_{\mathrm{eff}}}{U}\left(\frac{1}{t_{\mathrm{mig,L}}} - \frac{1}{t_{\mathrm{mig,EOF}}}\right) \qquad (1)$$

where l_{tot} and l_{eff} are the total and the effective capillary length and U is the applied separation voltage.

The obtained effective electrophoretic mobilitiy of the ligand was recalculated to the effective electrophoretic mobility at constant ionic strength (μ_{eff}) using the equation:

$$\mu_{eff} = \mu_0 - \left[\frac{8.204 \cdot 10^5}{(\varepsilon_r T)^{3/2}}\mu_0 + \frac{4.275}{\eta(\varepsilon_r T)^{1/2}}\right] \cdot \frac{\sqrt{I}}{1+50.29a(\varepsilon_r T)^{-1/2}\sqrt{I}} \quad (2)$$

where μ_0 is the mobility at zero ionic strength, I is the ionic strength of the background electrolyte, a is the ion size parameter, η, ε_r and T are the viscosity, the relative permittivity and the absolute temperature of the solvent, respectively.

3. Results and discussion

Determination of stability constant of ligand-ion complexes by affinity capillary electrophoresis is based on measurement of effective electrophoretic mobility of the ligand in the presence of complexing ion in the background electrolyte. In this study, the stability constant of dibenzo-18-crown-6 (ligand, L) complex with potassium ion (general metal ion, M^+) was determined by affinity capillary electrophoresis in mixed hydro-organic solvents at different water/methanol, water/ethanol and water/propan-2-ol ratios. In this method, the apparent stability constant of ML complex (K_{ML}) is obtained by nonlinear regression analysis of dependency of effective electrophoretic mobility of the ligand on the concentration of complexing ion in the background electrolyte using an equation:

$$\mu_{L,eff} = \frac{K_{ML}[M^+]\mu_{ML}}{1 + K_{ML}[M^+]} \quad (3)$$

where μ_{ML} is the mobility of the complex ML. If the ligand interacts with some other component of the background electrolyte, the mobility of the ligand in the absence of metal cation (μ_L^*) is taken into account and eq. (3) is modified to equation:

$$\mu_{L,eff} = \frac{\mu_L^* + K_{ML}^*[M^+]\mu_{ML}}{1 + K_{ML}^*[M^+]} \quad (4)$$

In this case, the obtained constant is called conditioned apparent stability constant. The measured dependencies fitted with the eq. (3) or eq. (4) are shown in Figure 1.

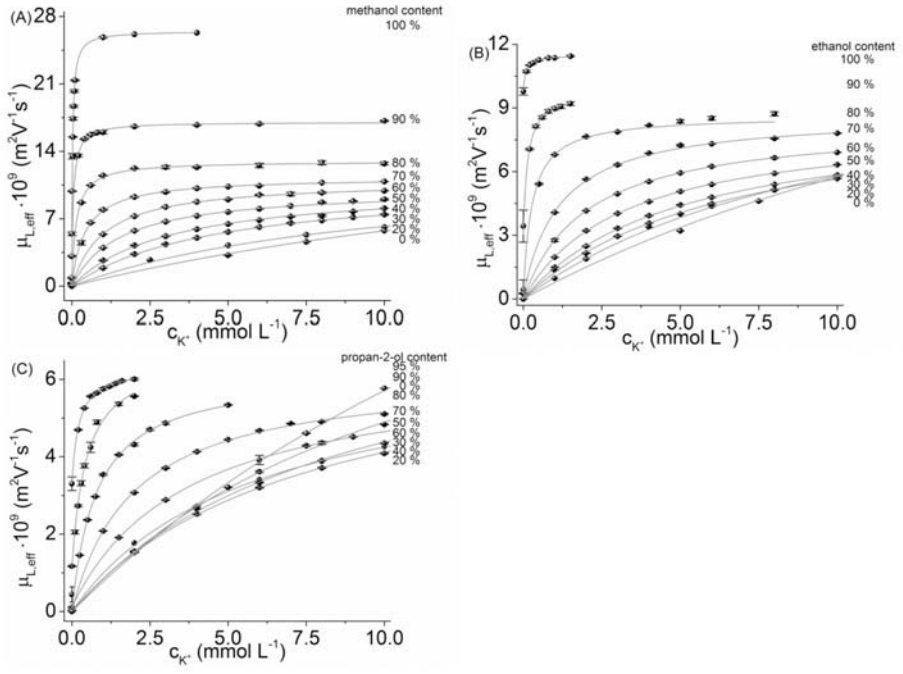


Fig. 1. Dependences of effective electrophoretic mobility of the ligand (dibenzo-18-crown-6) on concentration of potassium ions in background electrolyte at different ratios of methanol/water (A), ethanol/water (B), and propan-2-ol/water (C) mixed solvents.

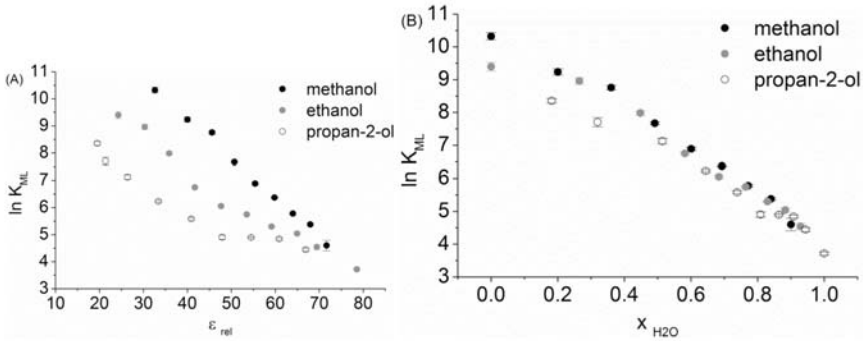


Fig. 2. Dependences of logarithm of stability constant on (A) relative permittivity of the water/methanol, water/ethanol and water/propan-2-ol mixed solvents, and on (B) molar fraction of water in these mixed solvents.

In order to find the solvent properties influencing the stability constant of the complex, the obtained $\ln K_{ML}$ (or $\ln K^*_{ML}$) were plotted as a function of relative permittivity of the mixed solvents, which represents their relevant physico-chemical parameter. Three different dependencies were obtained. The steepness of the curves decreases as the polarity of the organic solvent decreases (Fig. 2A). Plotting the dependency of the $\ln K_{ML}$ (or $\ln K^*_{ML}$) as a function of molar fraction of water in water/organic solvent mixture (Fig. 2B) shows that the K_{ML} obtained in studied water/organic solvent mixture are comparable up to molar fraction of water equal to 0.5. The lower molar fraction of the water in the solvent mixture results in different stability constants of the complex in the different mixtures of the same molar fraction of water.

4. Conclusion

Affinity capillary electrophoresis study of dibenzo-18-crown-6 complex with potassium ions in mixed hydro-organic solvents at different water/methanol and water/ethanol ratios has shown that the stability constant of this complex is strongly decreasing with increasing relative permittivity of the mixed solvents as well as with increasing molar fraction of water in these mixed solvents. On the other hand, the stability constant is almost independent of the above organic solvent used up to 50 molar % of water in the water/organic solvent mixture.

Acknowledgments

This work was supported by the Grant Agency of Charles University (GAUK 134215), the MEYS of CR (project SVV 260205), the Czech Science Foundation (grants nos. 13-17224S, and 13-21409P), and the Czech Academy of Sciences (project RVO 61388963).

References

[1] Izatt R.M., Pawlak K., Bradshaw J.S., Bruening R.L.: *Chem. Rev.* **91** (1991), 1721–2085.
[2] Chen Z., Weber S.G.: *TrAC Trends Anal. Chem.* **27** (2008), 738–748.
[3] Mironov G.G., Logie J., Okhonin V., Renaud J.B., Mayer P.M., Berezovski M.V.: *J. Am. Soc. Mass Spectrom.* **23** (2012), 1232–1240.
[4] Ehala S., Toman P., Makrlik E., Rathore R., Kasicka V.: *J. Solution Chem.* **41** (2012), 1812–1824.
[5] Dvorak M., Svobodova J., Benes M., Gas B.: *Electrophoresis* **34** (2013), 761–767.
[6] Shedlovsky T., Kay R.L.: *J. Phys. Chem.* **60** (1956), 151–155.
[7] Grunwald E., Berkowitz B.J.: *J. Am. Chem. Soc.* **73** (1951), 4939–4944.
[8] Bosch E., Rafols C., Roses M.: *Anal. Chim. Acta* **302** (1995), 109–119.

Application of accelerated solvent extraction for isolation of polycyclic aromatic hydrocarbons from smoked sausages

MÁRIA KOPUNCOVÁ[a, b, *]

[a] *National Agricultural and Food Centre, Food Research Institute, Department of Chemistry and Food Analysis, Priemyselná 4, 824 75 Bratislava, Slovakia* ✉ *kopuncova@vup.sk*
[b] *Comenius University, Faculty of Natural Science, Chemical Institute, Mlynská dolina, Ilkovičova 6, 842 15 Bratislava, Slovakia*

Keywords
accelerated solvent extraction
polycyclic aromatic hydrocarbons
smoked sausages
validation

Abstract
The aim of the present work was to develop and validate simplified procedure for isolation of benzo[*a*]anthracene, benzo[*b*]fluoranthene, benzo[*a*]pyrene and chrysene from smoked sausages. The novel method uses accelerated solvent extraction for the simultaneous isolation and clean-up of PAHs fraction in order to minimize consumption of solvents and chemicals, avoid losses of analytes and also save time. For the elimination of post-extraction clean-up step, activated silica gel as sorbent was applied directly to the accelerated solvent extraction extraction cell to retain fats and prevent the extraction of interfering compounds into PAHs fraction. The method passed through an in-house validation procedure and met all requirements set by the Regulation (EC) No 836/2011.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants which are produced during variety of combustion and pyrolysis processes from anthropogenic and natural sources [1, 2]. The International Agency for Research on Cancer included a number of them among substances with proven carcinogenic effect [3]. Incidence of PAHs was observed in majority of foodstuffs. Source of their contamination can be polluted environment but especially industrial processing methods based on thermal treatment of food such as drying, roasting, grilling and smoking, which permit direct contact between food and combustion products [1, 2].

In general, PAHs are not present individually but in complex mixtures. Because of the simplification of their analysis, benzo[*a*]pyrene has been used as a marker for the entire content of PAHs for a long time [1, 2]. In 2006, EU established maximum permitted concentrations of benzo[a]pyrene in foodstuffs [4] but, in addition, it strongly recommended also monitoring of other PAHs [5]. Later, the European Food Safety Authority reported that benzo[*a*]pyrene is not a suitable

indicator for the occurence of PAHs in food and proposed other reference compounds, such as benzo[*a*]anthracene, chrysene and benzo[*b*]fluoranthene [6]. Consequently, both maximum content of benzo[*a*]pyrene and the sum of all four compounds (PAH4) were established by the EC regulation No. 835/2011 [7]. For smoked meat products, the actual maximum benzo[*a*]pyrene content is 2 μg/kg and maximum content of PAH4 is 12 μg/kg [7].

One of the main difficulties related with the analysis of PAHs in smoked meat products is their high fat content (e.g. lipids, triglycerides, fatty acids). For that reason, the extraction of PAHs from these matrices is usually laborious and time consuming, and presents crucial step of the analysis [2]. Several articles were published in order to review the extraction methodologies which are currently applied in the determination of PAHs in food [2, 8]. Accelerated solvent extraction is one of the reported techniques, which has been successfully used for the extraction of PAHs from fatty matrices. It combines effect of elevated temperature and pressure to achieve very effective and fast extraction and entire process is fully automated, performed in minutes [9]. Moreover, accelerated solvent extraction allows the combination of extraction and pre-separation procedure in one analytical step [10, 11]. Thus, the aim of the present study was to develop and validate new method applying accelerated solvent extraction for simultaneous isolation and clean-up of PAH4 fraction from smoked sausages.

2. Experimental

2.1 Reagents and chemicals

Benzo[*a*]anthracene, benzo[*b*]fluoranthene, benzo[*a*]pyrene, and chrysene applied as standards were of analytical grade, purchased from Supelco in solid state. Standards were dissolved in acetonitrile to prepare standard solutions for spiking purposes. Poly(acrylic acid), partial sodium salt-graft-poly(ethylene oxide) was of analytical grade, purchased from Sigma Aldrich in solid state. Silica gel 40 was of analytical grade, purchased from Merck. Before the experiments, it was activated at 140 °C over 18 h. *n*-Hexane and acetone of analytical grade and acetonitrile and methanol of HPLC grade were purchased from Merck. Certified reference material Food Analysis Performance Assessment Scheme (FAPAS) Smoked Meat Product Ref. No. T0652QC with declared content of PAHs (benzo[*a*]anthracene 3.26 μg/kg, benzo[*a*]pyrene 0.54 μg/kg and chrysene 2.69 μg/kg) was purchased from the Food and Environment Research Agency (Sand Hutton, UK). Samples of smoked sausages, with fat content ranging between 48 and 52 %, were bought in local shops in Slovakia.

2.2 Instrumentation

For extraction, about 1 g of homogenized smoked sausage or reference material, mixed with the same amount of the drying material poly(acrylic acid), partial

sodium salt-graft-poly(ethylene oxide) was used. A 33-ml extraction cell was plunged with cellulose microfibre filter at the outlet. Then, 10 g of activated silica gel was put into the extraction cell. Subsequently, the sample was added and covered by the another microfibre filter.

The extraction was performed using accelerated solvent extraction equipment (ASE 350 Dionex) using *n*-hexane at 100 °C and 10 MPa at a static time of 10 min. The flush volume was 60% and the purge time 120 s [12]. Static cycle was accomplished three times. Then, the extract was evaporated in a water bath to dryness (40 °C) using a nitrogen stream. The residuum was dissolved in acetonitrile and analysed by HPLC with fluorescence detection.

HPLC analyses were carried out with Shimadzu equipment. The analytical separation was performed on Zorbax Eclipse XDB-C18 (100 mm×4.6 mm×1.8 μm) column connected to guard column Zorbax Eclipse PAH (12.5 mm×4.6 mm×5 μm) (Agilent) using isocratic elution with mobile phase acetonitrile/water 70:30 (v/v) at a flow rate 1.2 ml/min. A fluorescence detector operated at excitation/emission wavelength 260/410 nm for benzo[*a*]pyrene, 275/385 nm for benzo[*a*]anthracene and chrysene and 256/446 nm for benzo[*a*]fluoranthene.

The fat content determination was adopted from Lund et al. [11]. The presence of fats in extracts was confirmed by measuring UV-VIS spectra of extracts and visually from non-retained fats crystallised in vials after the evaporation of eluate.

3. Results and discussion

Despite of the indisputable advantages, accelerated solvent extraction is not selective extraction technique and it needs additional clean-up of extract, often more difficult and time consuming than the process of isolation. In the case of samples with high content of fat, such as smoked meat products, the most common clean-up procedure is gel permeation chromatography followed by the solid phase extraction. Jira [12] described and validated method included above mentioned techniques for analysis of PAHs consisting of from four to six rings.

However, also an integrated approach including isolation and clean-up of extract in one step is possible. First, Björklund et al. [10] studied possibilities of the lipid-free extraction of PCBs from fat containing matrices using five different fat retainers added directly into accelerated solvent extraction extraction cell. Later, Lund et al. [11] developed and validated accelerated solvent extraction method integrating extraction and fat retention in one single analytical step, which was applied for isolation of PAHs from fish products. However, they concluded that the experimental conditions can not be directly scaled to the different volumes of extraction cells, amount of fat retainers or sample matrices, respectively. Therefore, presented experiments were aimed at the development of concrete experimental conditions applicable for simultaneous extraction and clean-up of PAH4 fraction from smoked sausages.

Table 1
Recovery of PAH4 from reference materials using various fat-to-fat retainer ratios and numbers of static cycles.

Optimized parameters		Recovery[a] [%]	RSD [%]
FFR value	0.1	–[b]	–
	0.05	98	4.6
	0.03	77	6.8
Number of static cycles	1	15	15.2
	2	78	9.5
	3	98	6.7

[a] Determined in triplicate
[b] Recovery was not carried out due to presence of coloured impurities from spice

On the basis of above mentioned study carried out by Lund et al. [11], silica gel activated at 140 °C for 18 h was chosen as the most suitable fat retainer. The amount of sorbent, sufficient for the selective extraction of PAHs, was optimized using reference material, when the fat-to-fat retainer ratio was tested at three levels 0.03, 0.05 and 0.1 (Table 1). The fat-to-fat retainer ratio 0.1 was insufficient for complete removal of fats and other nonpolar impurities, such as carotenoids from spice and paprika. On the contrary, fat-to-fat retainer ratio 0.03 proved clean extract suitable for direct HPLC analysis, but the obtained recovery of PAH4 was low, indicating, that significant part of analytes was retained along with fats in layer of silica gel. Only ratio 0.05 was able to provide clean extract and also required recovery of PAH4.

Next parameter significantly affecting PAHs recovery was number of static cycles (Table 1). Extraction of the reference material was carried out with one, two and three static cycles. Only applying of three static cycles provided almost 100% recovery.

Regulation (EC) No 836/2011 [13] defines performance criteria for methods of PAHs analysis in food. In accordance with the regulation, parameters such as specifity, linearity, limit of detection and limit of quantification values, repeatability, reproducibility and recovery, were calculated.

Specifity was tested for coeluting impurities and spectral interferences during HPLC analysis. As it can be seen from Fig. 1, PAH4 were separated from each other while coeluting impurities were not observed.

Linearity of detector response was checked through calibration curves which were obtained by the linear regression of the peak area versus concentration of individual PAHs in injected solutions ranging from 0.1 μg/L to 15 μg/L. The calculated coefficients of determination were above 0.99 for all compounds (Table 2). Limit of detection and limit of quantification were calculated using the

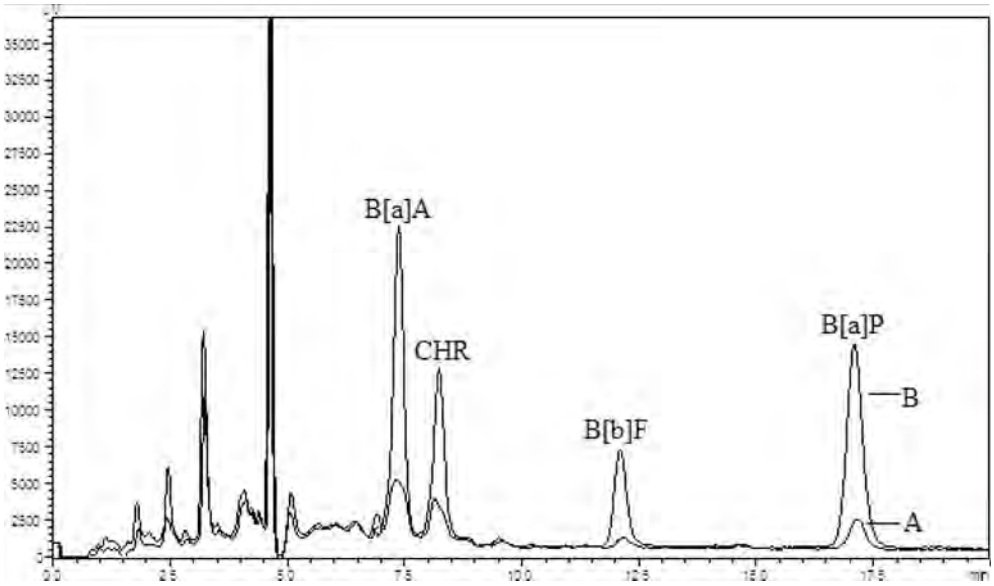


Fig. 1. Chromatographic record of studied PAHs in (A) smoked sausage, and (B) smoked sausage spiked with 5 μg/kg of each compound.

Table 2
Values of coefficient of determination, limit of detection, and limit of quantification for chromatographic determination of PAH4 calculated in the concentration range 0.10–15.00 μg/L.

Compound	R^2	*LOD* [μg/kg]	*LOQ* [μg/kg]
benzo[*a*]anthracene	0.99997	0.12	0.41
chrysene	0.99988	0.23	0.43
benzo[*a*]pyrene	0.99999	0.12	0.31
benzo[*b*]fluoranthene	0.99998	0.11	0.32

program LCSolution (Shimadzu) and, as it can be seen from Table 3, they met European Commission criteria [13], which set these values at 0.30 or 0.90 μg/kg, respectively.

Precision was assessed by the calculating of the Horwitz ratio in repeatability ($HORRAT_r$) and reproducibility ($HORRAT_R$) conditions. For evaluation of repeatability, commercial smoked sausages were analysed before and after spiking at individual PAHs content 0.5, 5 and 10 μg/kg in six replicates for each level. For reproducibility, analyses of commercial smoked sausages spiked at concentration level 5 μg/kg were carried out by the different analysts and also various (but of the same type) detectors were used. As follows from Table 3, $HORRAT_r$ and $HORRAT_R$ values ranged between 0.09 and 0.4 for all studied PAHs and thus, met the performance criterion currently set at value 2 (ref. [13]).

Table 3
Repeatability, recovery and reproducibility of PAH4 in smoked sausages.

Analyte	Repeatability			Recovery [%]	Reproducibility		
	Average content [μg/kg]	RSD_r [%]	$HORRAT_r$		Average content [μg/kg]	RSD_R [%]	$HORRAT_R$
benzo[*a*]anthracene	0.51±0.09	20.1	0.40	99±20			
	3.9±0.4	10.8	0.29	74±7	5.2±0.2	4.4	0.13
	8.9±0.7	8.1	0.25	81±7			
chrysene	0.47±0.09	19.2	0.38	98±19			
	5.1±0.3	5.7	0.16	102±5	6.5±0.7	10.1	0.3
	7.7±0.6	8.0	0.24	88±6			
benzo[*a*]pyrene	0.51±0.05	9.5	0.19	109±11			
	4.1±0.4	9.5	0.26	91±8	4.3±0.5	11.6	0.32
	109±11	6.0	0.19	89±6			
benzo[*b*]fluoranthene	0.50±0.05	10.3	0.20	108±11			
	4.3±0.2	3.4	0.09	84±3	4.1±0.2	4.5	11.6
	9.3±0.4	4.8	0.15	91±4			

The mean recovery of individual PAHs was then calculated. According to the performance criterion [13], recovery must be between 50 and 120%. As shown in Table 3, recovery achieved by the experiments ranged between 74±7 and 109±11% for all PAHs, which is in good accordance with the regulation.

Finally, the validated method was tested by the analysis of certified reference material FAPAS, reference number T0652QC Smoked Meat Product, with known content of benzo[*a*]anthracene, benzo[*a*]pyrene and chrysene, while the content of benzo[*b*]fluoranthene was not declared. As shown in Table 4, three PAHs were

Table 4
Results of analysis of reference certified material FAPAS matrix Ref. No. T0652QC Smoked Meat Product.

Analyte	Average value calculated on the basis of experimental data [μg/kg]	Range[a] for $\|z\|\leq 2$ [μg/kg]
benzo[*a*]anthracene	4.00±0.50	1.83–4.70
chrysene	3.20±0.20	1.51–3.88
benzo[*a*]pyrene	0.36±0.08	–
benzo[*b*]fluoranthene	0.45±0.04	0.30 0.78

[a] Range for $|z|\leq 2$ is the concentration range within the limits of ±2 *z* scores. The assigned values and their range were established from the proficiency test data and are suitable for use by laboratories as a fit-for-purpose quality control measure.

determined within the declared range set by the producer of material. The content of benzo[*b*]fluoranthene was quantified despite the fact that its content was not declared.

4. Conclusions

On the basis of experimental obtained data, it is possible to postulate that accelerated solvent extraction is extraction technique suitable for the isolation of PAHs from smoked meat sausages. Moreover, after selecting of optimum fat/silica gel mass ratio for removal of impurities extracted along with PAHs, accelerated solvent extraction can be advantageously used for the simultaneous isolation and clean-up of the PAHs fraction and allows direct analysis of extract by HPLC. Described procedure significantly shortens duration of analysis, reduces handling the sample and minimize consumption of used chemicals. As revealed presented results of in-house validation procedure, the new method meets all requirements set by the Regulation (EC) No 836/2011 [13].

Acknowledgments

This work is the result of a project supported by the Science and Technology Assistance Agency of the Slovak Republic under contract No. APVV-0168-10.

References

[1] Guillén M. D., Sopelana P.: Polycyclic aromatic hydrocarbons in diverse foods. In: *Food Safety: Contaminants and Toxins.* D'Mello, J. P. F. (edits). Wallingford, CABI Publishing, 2003, p. 175–198.
[2] Plaza-Bolaños P., Frenich A.G., Vidal J L.M.: *J. Chromatogr.* **A** 1217 (2010), 6303–6326.
[3] International Agency for Research on Cancer (IARC): Some non-heterocyclic polycyclic aromatic hydrocarbons and some related exposures. In: *Monographs on the Evaluation of Carcinogenic Risks to Humans.* IARC, Lyon, 2010.
[4] European Union (EU), Commission Regulation (EC) No 1881/2006, *Off. J. Eur. Union* **L364** (2006), 5–25.
[5] European Union (EU), Commission Recommendation No 2005/108/EC, *Off. J. Eur. Union* **L34** (2005), 43–45.
[6] Polycyclic aromatic hydrocarbons, Scientific opinion of the panel on contaminants in the food chain, *EFSA Journal* **724** (2008), 1–114.
[7] European Union (EU), Commission Regulation (EC) No. 835/2011, *Off. J. Eur. Union* **L215** (2011), 4–8.
[8] Purcaro G., Moret S., Conte L.S.: *Talanta* **105** (2013), 292–305
[9] Mustafa A., Turner C.: *Anal. Chim. Acta* **703** (2011), 8–18.
[10] Björklund E., Müller A., von Holst C.: *Anal. Chem.* **73** (2001), 4050–4053.
[11] Lund M., Duedahl-Olesen L., Christensen J.H.: *Talanta* **79** (2009), 10–15.
[12] Jira W.: *Eur. Food Res. Technol.* **218** (2004), 208–212.
[13] European Union (EU), Commission Regulation (EC) No 836/2011, *Off. J. Eur. Union* **L215** (2011), 9–16.

The use of selected analytical techniques for pollutants determination in the environmental samples collected from the Arctic regions

KLAUDIA KOSEK*, ŻANETA POLKOWSKA

Department of Analytical Chemistry, Faculty of Chemistry, Gdansk University of Technology, 11/12 Narutowicza St., Gdansk 80-233, Poland ✉ *klaudia_kosek@wp.pl*

Keywords
analytical techniques
Arctic environment
climate change
long range transport

Abstract
The paper concern importance of the use of analytical techniques for pollutants determination in the Arctic based on the example of total organic carbon, phenols and formaldehyde determination and measurement of pH and electrical conductivity parameters in surface water samples collected from the Revelva catchment.

1. Introduction

The Arctic encompasses the lands and waters north of 60° latitude including approximately 20 million km^2 of ocean, all of Iceland and Greenland, the northern reaches of Norway, Finland, Russia, Canada north of the southern shore of Hudson Bay and Alaska north of the panhandle in the United States [1].

The Svalbard Archipelago is located at the NW limit of the European continental shelf between 76.50–80.80 °N and 10–34 °E [2]. It is one of ten the most glaciated areas in the Arctic. The total area of Svalbard is 62 800 km^2 and glaciers cover 36 600 km^2 (about 60%) [3]. During the past decade, the Arctic has undergone dramatic change and it is not consider a pristine region any more. An interest in the processes of transport of wide range of pollutants and them fate in polar regions located distantly from industrial centers is still increasing. The current analytical techniques enabling conducting studies prove that the Arctic has become an area of highly intensive anthropopressure and it is possible to recognize considerably low but still harmful and persisting levels of pollutants concentration present there [4, 5].

Chemical substances of anthropogenic origin that appear in polar regions are often considered as persistent organic pollutants-POPs (e.g. PAH, PCB, DDT), metals, phenols, formaldehyde and radioactive isotopes. Accumulation of these compounds in northern latitudes is a well-documented phenomenon and it is

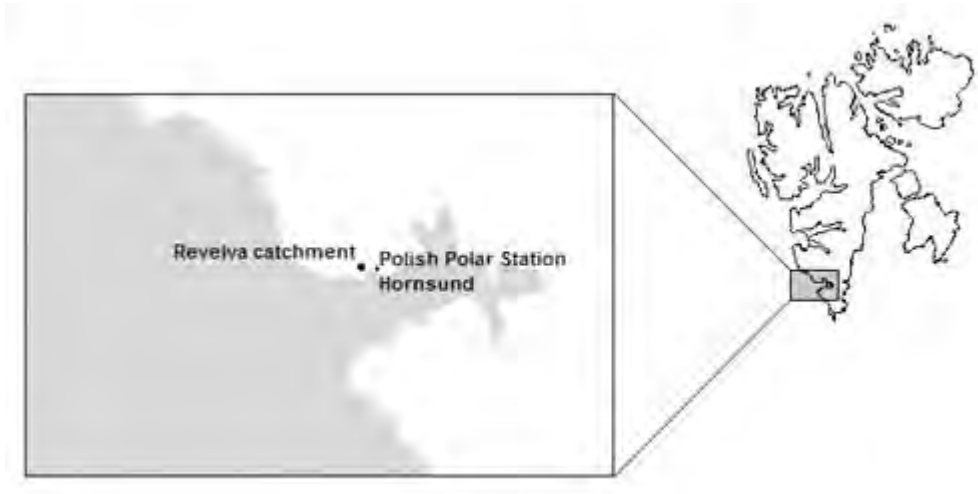


Fig. 1. The map of Svalbard taking into account location of the Revelva catchment.

thought to occur for a variety of reasons. In a process known as a global distillation, prevailing ocean and wind currents bring contaminants to the Arctic where they are subsequently trapped in all kinds of environmental reservoirs by the cold climate. This process is often called the grasshopper effect, as chemicals repeatedly evaporate and condense as long as they reach the Arctic. Besides the long-range transport of pollutants, contaminants appearing in Svalbard are also the consequence of some small local sources such as military installations, industrial outlets, settlements and ships [6].

2. Experimental

2.1 Study area

The study area covered the southern part of Wedel-Jarlsberg Land-Spitsbergen (Svalbard Archipelago). The primary study object was the Revelva catchment in the vicinity of Polish Polar Station at Hornsund (Fig. 1). The main river (Revelva) is fed both directly by atmospheric precipitation, snow melt water streams and a river originating from the Arie Glacier. Revelva drains into the bay of Ariebukta in the south, forming an estuary. In the upper part of the catchment, the main streams originate from the slopes of Eimfjellet (640 m asl) and Skålfjellet (635 m asl). The Revelva catchment has only one small glacierised part but past glacial activity has left traces in its upper part. The bottom part of the Revelva valley is an elevated marine terrace, with abrasion stacks. On the terrace, areas of patterned ground and contemporary storm ridges have been formed. The diversity of the catchment landscape provides an ideal setting for a comprehensive study of processes of pollutants deposition in different parts of the abiotic environment [7].

Table 1
Technical specifications and metrological characteristics of analytical techniques used in chemical analysis.

Parameter	*LOD*[a]	*LOQ*[a]	Measurement equipment and method
TOC [mg/L]	0.030	0.100	Total Organic Carbon Analyzer TOC-VCSH/CSN, SHIMADZU (method of catalytic combustion (oxidation) with the application of the NDIR detector)
Electrical conductivity [μS/cm]	–	–	Conductometr OK-102/1, RADELIKS (conductivity method)
pH	–	–	pHmetr CX-401 ELMETRON (potentiometric method)
Sum of phenols [mg/L]	0.025	0.075	Absorbance measured at 495 nm using Spectroquant Pharo 100
Formaldehyde [mg/L]	0.02	0.06	Absorbance measured at 585 nm using Spectroquant Pharo 100

[a] The limit of detection (*LOD*) and the limit of quantitation (*LOQ*) were calculated based on the standard deviation of the response (s) and the slope of the calibration curve (b), according to the formulas: $LOD = 3.3(s/b)$, $LOQ = 10(s/b)$

2.2 Sampling

Surface water samples were collected from the Revelva catchment (9 water samples collected from the Revatnet Lake and 1 sample collected from the river) every summer from July 2010 to July 2013 in the vicinity of the Polish Polar Station at Hornsund. Samples were collected using a manual sampling technique and stored under refrigerated conditions.

2.3 Analytical Methods

The samples were transported to the laboratory of the Department of Analytical Chemistry (Gdansk University of Technology) in tightly closed bottles and stored at a temperature of 4 °C (to avoid the loss of analytes) until chemical analysis. Information on the analytical techniques used to determine total organic carbon (TOC), pH, electrical conductivity, sum of phenols and formaldehyde with their metro-logical characteristics are given in table 1.

The demineralized water Mili-Q type obtained from apparatus Mili-Q® Ultrapure Water Purification Systems (Milipore® production) was used in chemical analysis.

3. Results and discussion

Total organic carbon (TOC) is the amount of carbon bound in an organic compound and it is the sum of suspended and dissolved organic carbon. This constitutes a huge range of compounds with a variety of properties. TOC is released to environment from natural (metabolism, excretion and deposition of organisms) and anthropogenic (sewage treatment plants, farm slurry, silage runoff) sources. As well as TOC, in polar environmental samples formaldehyde and sum of phenols may be found. They constitute a significant amount of pollutants transported to the Arctic from lower latitudes [8]. In the Fig. 2, there are shown the average values of sum of phenols and formaldehyde concentration levels observed in collected samples from summer 2010 to 2013.

Significantly high concentration levels of formaldehyde in 2012 may be caused due to increased emission of pollutants from industrialized areas compared with other years and its transport to the Arctic. It can be also observed an increasing trend in phenols concentration levels over 4 years that may be an important indicator of climate change.

The hydrochemical studies of polar areas were carried out over many years in the surroundings of Polish Polar Station at Hornsund, also in the catchment of

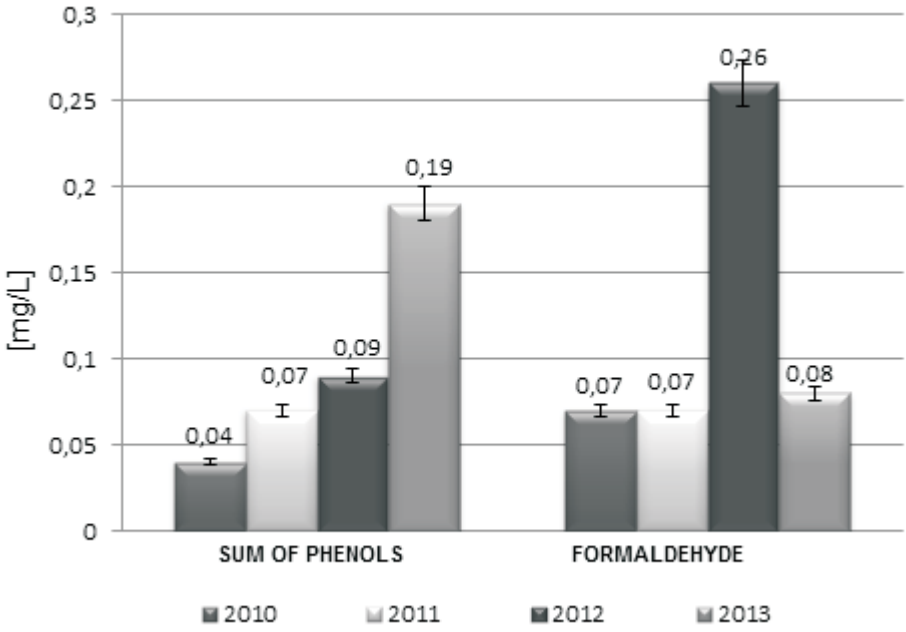


Fig. 2. The average values of phenols and formaldehyde concentration levels in samples collected every summer from 2010 to 2013 from Revelva catchment.

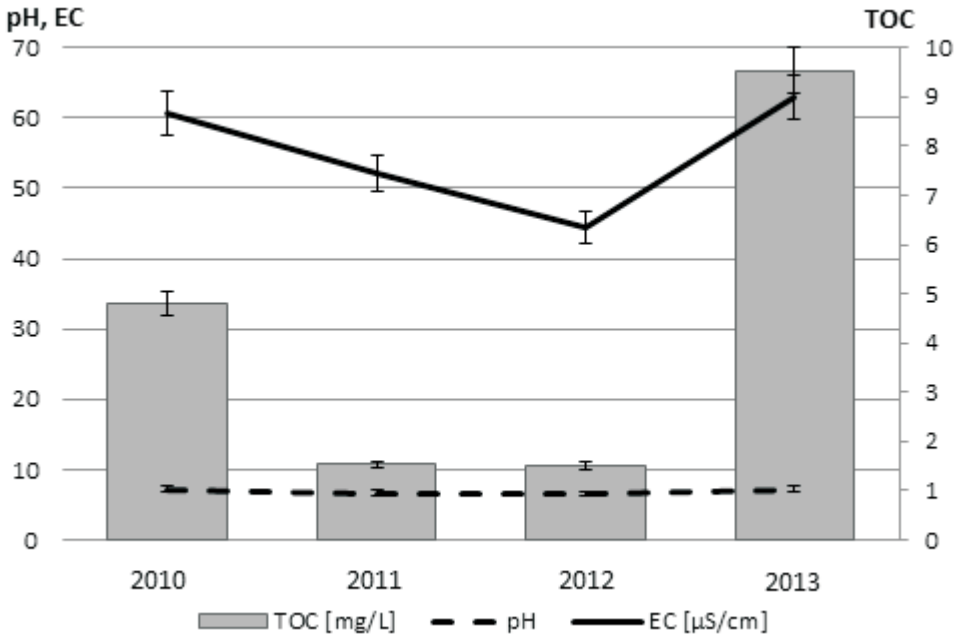


Fig. 3. The average values of pH, electrical conductivity and total organic carbon in analysed samples.

Revelva and demonstrated high hydrochemical variability. In these studies, it may be observed that surface water samples were characterised by slightly acidic pH (Fig. 3.).

The highest determined levels of total organic carbon and electrical conductivity were observed in samples collected in summer 2013. The average value of total organic carbon this year was 9.54 mg/L and electrical conductivity increased to 63.0 µS/cm. The lowest average values of these parameters were observed in two previous years. Summing up, TOC and electrical conductivity parameter are strongly related: higher conductivity reflects higher sample pollution. However higher level of EC may also suggests the presence of other chemical compounds in analysed samples which do not contain organic carbon.

In the Fig. 4, there are shown correlations between sum of phenols, formaldehyde and total organic carbon. The correlation between TOC and sum of phenols is visible on the graph below (Fig. 4A). It may be stated that with the increase of sum of phenols concentrations, total organic carbon concentration levels also increase. Another situation is presented on the next graph (Fig. 4B). Even though the concentration levels of TOC are still higher than formaldehyde content, the correlation between these compounds is altered. For instance, in summer 2013, concentration levels of TOC reached almost 10.0 mg/L and formaldehyde content in surface water samples were practically insignificant that

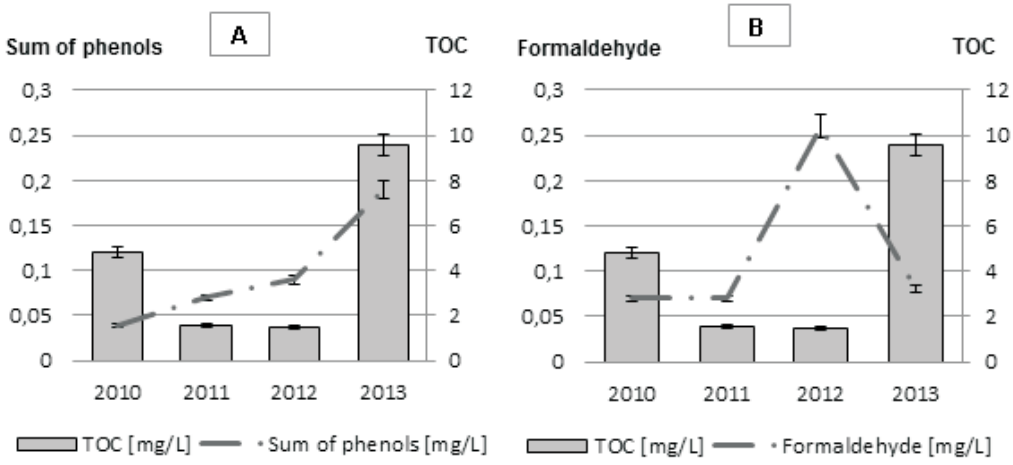


Fig. 4. The average values of sum of phenols, formaldehyde and total organic carbon in analysed samples.

year. It can be explained by the occurrence of other carbon compounds in the Arctic such as black carbon (BC) which emitted into the atmosphere, warms the Arctic in two days or methane.

Water reservoirs contain carbon in various forms. In the ocean, dissolved organic carbon comes from decaying biological material. Dissolved inorganic carbon includes carbon dioxide and other simple molecules and ions containing carbon. Both organic and inorganic are also present in particulates. Lakes and rivers are responsible for most of the direct transport of carbon from land to ocean. The Arctic holds 36% of the world's lake surface area and accounts for 10% of global river discharge to the ocean. Taking the same proportions of estimates of global freshwater carbon releases gives an estimate for the Arctic of 25–54 million tonnes of carbon from lakes each year and 15-30 million tonnes from rivers [10].

4. Conclusions

According to actual scientific knowledge, a wide range of pollutants are transported and distributed throughout the Arctic via natural distribution pathways including the atmosphere, transpolar ice drift, ocean currents and rivers [9]. The results of analyses of surface water samples from the Revelva catchment show an increasing trend in pollutants concentration levels over the years. It may suggests that each year the emission of contaminants from lower latitudes is bigger and more of them are deposited in the Arctic what may serve as early warning signal of expected climate change. During the past few decades, monitoring and fate studies of pollutants in the Arctic have been conducted mainly

in the marine environment and the surrounding coastal zones. Only a few comprehensive studies exist on the fate and distribution of pollutants in the terrestrial environment and freshwater reservoirs [9]. Therefore, it is important to continue monitoring contaminants concentration levels of whole area and to develop current analytical techniques for all kinds of xenobiotics determination because even low pollutants concentration levels in polar regions may suggest a significant contamination of studied area.

References

[1] Suk W., Avakian M., Carpenter D., Groopman J., Scammell M., Wild C.: *Environ. Health Persp.* **112** (2004), 1130–120.
[2] Błaszczyk M., Jania J.A., Hagen J.O.: *Pol. Polar Res.* **30** (2009), 85–142.
[3] Hagen J.O., Kohler J., Melvold K., Winther J.G.: *Polar Res.* **22** (2003), 145–159.
[4] Kozak K., Polkowska Ż., Ruman M., Kozioł K., Namieśnik J.: *Trends Anal. Chem.* **50** (2013), 107–126.
[5] Ruman M., Kozak K., Lehmann S., Kozioł K., Polkowska Ż.: *Ecol. Chem. Eng. S.* **19** (2013), 571–584.
[6] Wania F.: *Environ. Sci. Technol.* **37** (2003), 1344–1351.
[7] Birkenmajer K.: *Polish Academy of Science, Committee on Polar Research and Silesian University* (1990), 42 pp.
[8] Kosek K., Lehmann S., Gajek G., Kociuba W., Franczak Ł., Polkowska Ż.: Morphometric parameters of the Renardbreen as imporant factors determining the spatial distribution of chemical compounds on the glacier surface (Bellsund, Svalbard). In: *New Perspectives in Polar Research.* Migała K., Owczarek P., Kasprzak M., Strzelecki M.C. (edits.). Wrocław 2014, p. 97–109.
[9] AMAP, 2011. *Combined Effects of Selected Pollutants and Climate Change in the Arctic Environment.* By: R. Kallenborn, K. Borgå, J.H. Christensen, M. Dowdall, A. Evenset, J.Ø. Odland, A. Ruus, K. Aspmo Pfaffhuber, J. Pawlak and L.-O. Reiersen. Arctic Monitoring and Assessment Programme (AMAP), Oslo. 108 pp.
[10] AMAP, 2009. AMAP 2009 *Update on Selected Climate Issues of Concern. Arctic Monitoring and Assessment Programme*, Oslo. v+15pp.

The use of INCAT device for the analysis of volatile components in urine samples

Peter Kotora[a,*], Dušana Hudecová[a], Viktória Ferenczy[a], Alexandra Hengerics Szabó[a], Jaroslav Blaško[a], Róbert Kubinec[a], Ngô Manh Thắng[b]

[a] *Comenius University in Bratislava, Faculty of Natural Sciences, Institute of Chemistry, Ilkovičova 6, 842 15 Bratislava, Slovakia* ✉ *kotora@fns.uniba.sk*
[b] *Department of Physical and Analytical Chemistry, Faculty of Chemical Engineering, HCMC University of Technology, 268 Ly Thuong Kiet, District 10, HCM City, Vietnam*

Keywords
GC-MS
INCAT
needle trap
preconcentration
urine

Abstract
Work was focused on the development of INCAT, a solventless microextraction technique with adsorbents inside a steel needle. The device was used for the extraction and preconcentration of volatile compounds, subsequently analysed by gas chromatographic methods. The sorption characteristics of INCAT devices with different sorbents were compared and the device with sorbents Chromosorb W + 20% SE-54, Carbopack X and Carboxen 1000 (in 1:1:1 v/v ratio) was applied for the analysis of volatile organic compounds in urine samples of patients with metabolic diseases.

1. Introduction

Sample preparation represents one of the cornerstones of analytical procedures. Its main requirements are the extraction of analytes from a complex biological matrix and their transformation into forms compatible with the analytical technique used while also removing the interfering matrix components. When metabolites are present in low concentrations in the samples, preconcentration is often needed in addition to extraction to make the analyte determination with the selected technique possible. The sample preparation should be simple and universal because every sample preparation step causes losses of the analytes. The methods for the extraction of highly polar analytes from aqueous samples have particularly low yields of extraction. Currently the development of methods allowing the reduction or elimination of sample preparation steps needed is preferred. Minimisation of sample preparation steps also reduces the time and cost of analyses [1]. The most common solventless extraction methods are headspace techniques, direct aqueous injection (DAI), solid phase microextraction (SPME), needle trap extraction etc. Needle traps represent a robust and promising tool for sample pretreatment combining the advantages of SPE and SPME [2]. One of their main advantages is their robustness compared to SPME and that they do

not need a special desorber, they fit into the heated GC injector port [3]. The use of needle traps with a single adsorbent was proved in the field of environmental monitoring [4–7]. The analysis of complex samples containing analytes in a wide range of polarity and volatility need a multilayered needle trap with different adsorbents. Inside needle capillary adsorption trap (INCAT) is a needle trap developed by Kubinec et al [5, 8, 9]. This device is more robust than most needle traps or SPME and gives comparable results [9].

Modern medicine is based on the idea that the analysis of blood, urine, tissues and other body fluids and gases can give insights and information needed for the diagnosis of diseases, for the monitoring of the diseases or their therapy [10]. New diagnostic methods must not represent any risk for the patient even when frequently repeated and have to provide more information than is obtained from the routine blood and urine tests.

Gas chromatography with FID or MS detection still represents the golden standard in the analysis of volatile metabolites. Detection by MS/MS is still relatively rarely used because of the high variability of settings and unavailability of commercial MS/MS spectral libraries.

This work was focused on the development of an INCAT device enabling the preconcentration and extraction of volatile compounds in urine samples and their subsequent GC-MS analysis. The aim was not the quantitative analysis of volatile compounds present in urine samples, but the development of a new method enabling the determination of volatile compounds in this matrix. Quantification and statistical analysis of the obtained results will be the topic of another work.

2. Experimental

2.1 Materials

For the analysis of volatile metabolites in urine samples active mode of sampling was used. The samples were preconcentrated by microextraction technique INCAT. INCAT devices were prepared from stainless steel needles (1.3/1.1 and 1.1/0.9 mm o.d./i.d., 80 mm long), sorbents Chromosorb W + 20% SE-54, Carbopack X and Carboxen 1000 (in 1:1:1 v/v ratio). Sampling of urine samples was carried out using a Neuberger KNF Laboport N86KT.18 membrane pump by drawing a constant volume of gaseous samples 500 mL.

2.2 Instrumentation

Gas chromatographic analyses were performed using a Network GC System 6890N (Agilent Technologies) gas chromatograph with a 5973N MSD (Agilent Technologies) MS detector operating in SCAN mode. Chromatographic separation was carried out on a 60 m × 0.32 mm × 5 μm DB-1 capillary column. Samples were injected in splitless mode with splitless time set to 3 minutes. The better

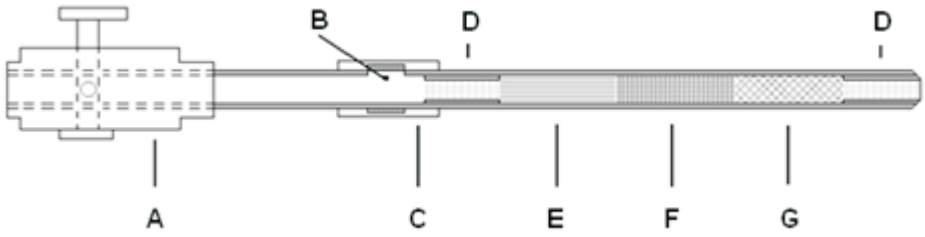


Fig. 1. Scheme of INCAT device: (A) sampling valve, (B) side hole, (C) teflon and silicone sealing, (D) frit with sintered glass beads, (E– G) adsorbents.

desorption of the analytes from the INCAT device was achieved by using a special metal liner in the injector [5]. Helium was used as a carrier gas with constant pressure at the injector 121 kPa. Injector temperature was set to 280 °C. The oven temperature was set to 20 °C for 3 minutes and gradually increased to 280 °C at a rate of 10 °C/min and was held for 13 minutes.

3. Results and discussion

INCAT device was prepared from an 80 mm long stainless steel needle with outer diameter 1.3 mm and inner diameter 1.1 mm. A 0.6 mm diameter side hole was drilled into this needle 30 mm from its end and a thread was cut into the end. Into the front part a 5 mm long frit was inserted which was previously prepared from a 1.1/0.9 mm stainless steel needle filled and sintered with 0.125–0.2 mm glass beads. The needle tip was made round and smooth to avoid septum tearing during sample injection. The device was then filled with the selected adsorbents and a second frit was inserted between the adsorbents and the side hole to fix the position of the adsorbents. The side hole served for the better desorption of the analytes from the INCAT device as its position was chosen thus the carrier gas could flow through it in the injector of the gas chromatograph. During sampling the side hole must be covered to prevent the device from the collection of outdoor air. That is why tubings from teflon (because of its inertness) and silicone (because of its good sealing ability) were used to cover the side hole during sampling. To the end part of the INCAT device a closing valve was attached. The scheme of the INCAT device is shown on Fig. 1.

The aim of this work was the development of an INCAT device enabling the sampling of compounds in a wide range of volatility. The sorption-desorption characteristics of INCAT devices were tested by filling the devices with different adsorbents and analysing a mixture of *n*-alkanes C_6–C_{16}. Various types of adsorbents were tested and based on the obtained results Chromosorb W + 20% SE-54, Carbopack X and Carboxen 1000 were chosen as presented on Fig. 2.

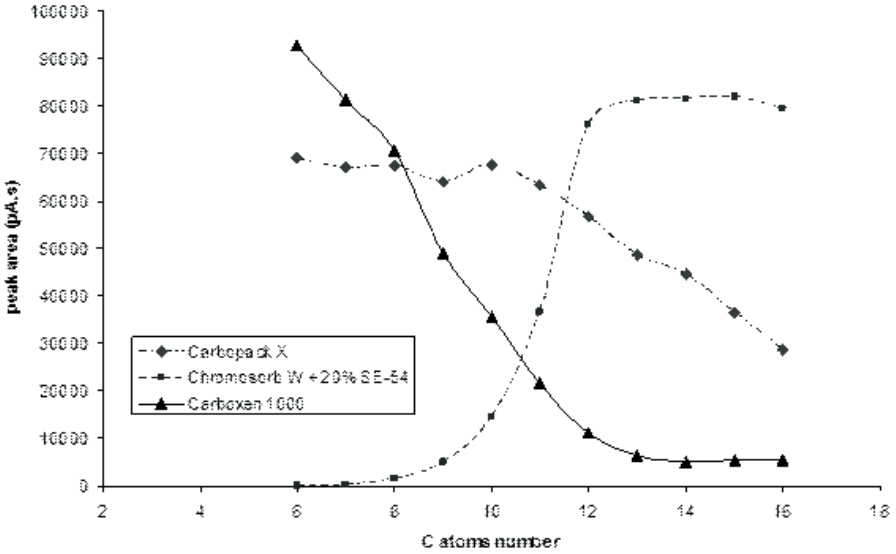


Fig. 2. Peak areas of alkanes C_6–C_{16} after the desorption from INCAT devices packed with different sorbents.

As can be seen from Fig. 2, each INCAT device has different sorption characteri-stics for the tested *n*-alkane mixture. The INCAT device containing only Carboxen 1000 enables the quantitative sorption of all alkanes, but the higher alkanes cannot be desorbed. The INCAT device with Chromosorb W only does not allow the sorption of alkanes $<C_{12}$, but all the higher are desorbed quantitatively. The INCAT device containing three different adsorbents in the same proportion was the best option for the sorption-desorption of the widest range of alkanes.

10 mL of urine samples were transferred into a 60 mL vial. This ratio ensured enough space for the forming foam without its connection with the INCAT device. As can be seen from Fig. 3, the INCAT device was pierced through the vial septum together with a capillary securing the supply of pure nitrogen. Using nitrogen as an inert gas served for the reduction of contamination during urine sampling by active sampling. The amount of collected gas phase was 500 mL.

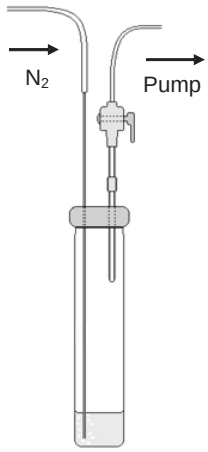


Fig. 3. Sampling of volatile analytes from urine samples by INCAT device.

Figure 4 shows a chromatogram of a urine sample from a patient with a metabolic disease. As we can see ketonic compounds are present at higher concentration in this sample compared to the urine sample of a healthy individual.

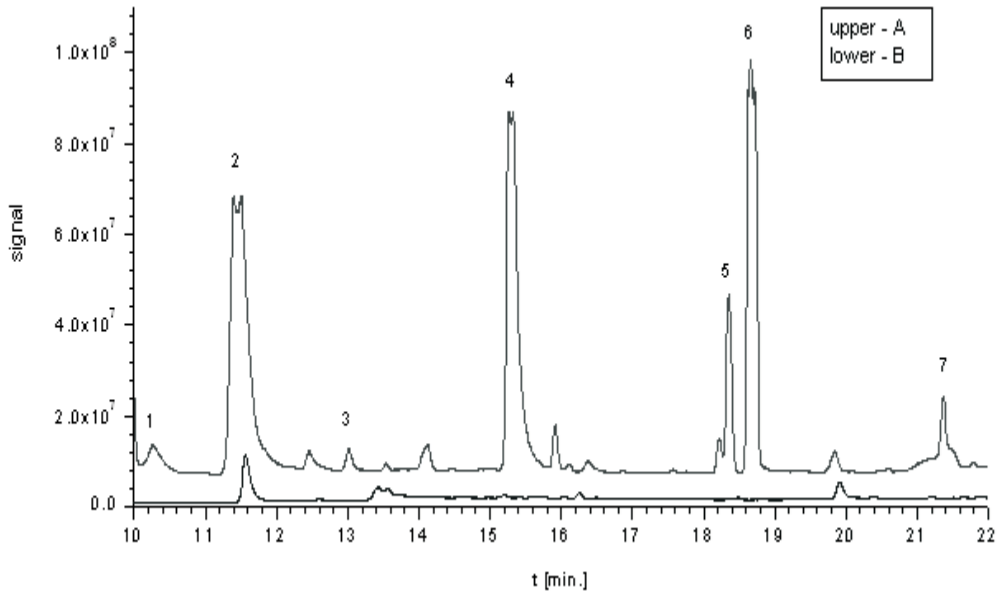


Fig. 4. Chromatogram of a urine sample from (A) a patient with metabolic disease and (B) a healthy individual: (1) ethanol, (2) acetone, (3) acetic acid methyl ester, (4) buthan-2-one, (5) pentan-2-one, (6) pentan-3-one, (7) hexan-3-one.

4. Conclusions

This work was focused on the development of an INCAT device enabling the preconcentration and extraction of volatile compounds in urine samples and their subsequent GC-MS analysis. By selecting adsorbents ensuring good sorption--desorption characteristics of the INCAT device in a wide range of volatility we developed an INCAT device enabling the sampling, preconcentration, extraction and injection of volatile compounds in urine into the GC system. The results prove that by using the developed sample preparation method volatile metabolites indicating diseased health state can be analysed without the need of derivatisation or sample modification.

Acknowledgments

This publication is the result of the project implementation: Comenius University in Bratislava Science Park supported by the Research and Development Operational Programme funded by the ERDF. Grant number: ITMS 26240220086.

References

[1] Kataoka H., Ishizaki A., Nonaka Y., Saito K.: *Mass Spectrom. Rev.* **26** (2007), 51–78.
[2] Lord H.L., Zhan W., Pawliszyn J.: *Anal. Chim. Acta* **677** (2010), 8–29.
[3] Eom I.Y., Pawliszyn J.: *J. Sep. Sci.* **31** (2008), 2283–2287.
[4] Eom I.Y., Niri V.H., Pawliszyn J.: *J. Chromatogr. A* **1196–1197** (2008), 10–14.

[5] Jurdáková H., Kubinec R., Jurčišinová M., Krkošová Ž., Blaško J., Ostrovský I., Soják L., Berezkin V.G.: *J. Chromatogr. A* **1194** (2008), 161–164.
[6] Niri V.H., Eom I.Y., Kermani F.R., Pawliszyn J.: *J. Sep. Sci.* **32** (2009), 1075–1080.
[7] Koziel J.A., Odziemkowski M., Pawliszyn J.: *Anal. Chem.* **73** (2001), 47–54.
[8] Kubinec R., Berezkin V.G., Górová R., Addová G., Mračnová H., Soják L.: *J. Chromatogr. B* **800** (2004), 295–301.
[9] Přikryl P., Kubinec R., Jurdáková H., Ševčík J., Ostrovský I., Soják L., Berezkin V.: *Chromato-graphia* **64** (2006), 65–70.
[10] Risby T.H.: Current status of clinical breath analysis. In: *Breath Analysis for Clinical Diagnosis and Therapeutic Monitoring.* Amann A., Smith D. (edits.). Singapore, World Scientific Publishing 2005, p. 251–265.

Preliminary testing of dispersive liquid–liquid microextraction for extraction of estradiol from human urine

Elena Kupcová*, Katarína Reiffová

Pavol Jozef Šafárik University, Faculty of Science, Department of Analytical Chemistry, Moyzesova 11, 040 01 Košice, Slovakia ✉ *elena.kupcova@gmail.com*

Keywords
chemical and densitometric detection
dispersive liquid–liquid microextraction
estradiol
thin-layer chromatography
urine

Abstract
A new microextraction protocol was tested for the isolation of 17β-estradiol, a main human estrogen, from urine. Dispersive liquid–liquid microextraction (DLLME) was selected owing to its simplicity, rapidity and preconcentration factor, due to complex character of urine sample and low concentration of analyte. In order to attain efficient extraction, several parameters, including type and volume of extraction solvent and dispersive solvent, salt addition, extraction time, and centrifugation conditions, were studied. The optimal conditions were achieved after rapid injection of methanol (dispersive solvent) and tetrachloroethane (extraction solvent) mixture (9:1; v/v) into urine sample with addition of 1 M NaCl, thus achieving sample pH ≥ 10. Efficiency of DLLME procedure was evaluated by thin-layer chromatography with chemical and densitometric (λ = 430 nm) detection. Method provided linearity within concentration range 0.1–1.0 mg/mL (R^2 = 0.9965) and *LOD* = 0.1 mg/mL. Proposed method was applied to the analysis of free estradiol in real human pregnancy urine.

1. Introduction

17β-estradiol (estradiol) is main human estrogen responsible for development and function of female gonadal system [1]. This sex hormone is crucial for regulation of menstrual cycle and maintenance of pregnancy in women; in much lesser extent takes part in various biochemical processes within the organism in both males and females [2]. Estrogens originate in steroidogenesis with estradiol being the main product and most potent human estrogen. Mean daily production of estradiol in women is 1.7–4.6 μg/day and usually elevates to 340–600 μg/day during pregnancy [3]. It is released into the bloodstream and transported to tissues with specific estrogen receptors which enable estrogen action [4]. Estradiol is excreted in urine mostly in the form of conjugates with glucuronic or sulphuric acid, but still with small portion remaining in free form. High levels of estrogens are associated with breast and ovary cancer [5, 6] in females and prostate cancer in males [7]; due to this fact, analysis of estrogens is very popular

mainly in environmental water samples [8, 9]. Liquid chromatographic methods, primarily HPLC, are the most commonly used for quantification of estrogens owing to their high sensitivity [10, 11]. Still, sample pretreatment remains obligatory, mainly in biological sample analysis, due to complex character of the sample and low concentration of estrogens (ng/L).

Dispersive liquid-liquid microextraction (DLLME) is a novel microextraction technique introduced in 2006 [12]. It is a simple and fast sample preparation technique offering high preconcentration factors and recovery. DLLME consists of ternary solvent system in which a dispersive solvent causes dispersion of an extraction solvent into the sample thus enlarging the phase boundary and allowing rapid extraction of desired analyte. A mixture of extraction and dispersive solvents is rapidly injected into the aqueous sample with a syringe leading to the formation of tiny droplets. After centrifugation, sedimented organic drop of extraction solvent containing analyte of interest is removed with a microsyringe and injected into compatible analytical system (Fig. 1) [13].

DLLME was introduced as an extraction technique for organic compounds from water samples and has later developed to be suitable for extraction of analytes from more complex samples, including biological [14]. Few DLLME protocols for extraction of estrogens have been described [15–19]; however all of them were limited to water samples. This work aims to test applicability of DLLME for estradiol extraction from urine samples. Thin-layer chromatography (TLC) was used for preliminary testing of DLLME due to the possibility of simultaneous analysis of larger group of samples that allows relatively fast evaluation of numerous samples despite its lower sensitivity when compared to HPLC.

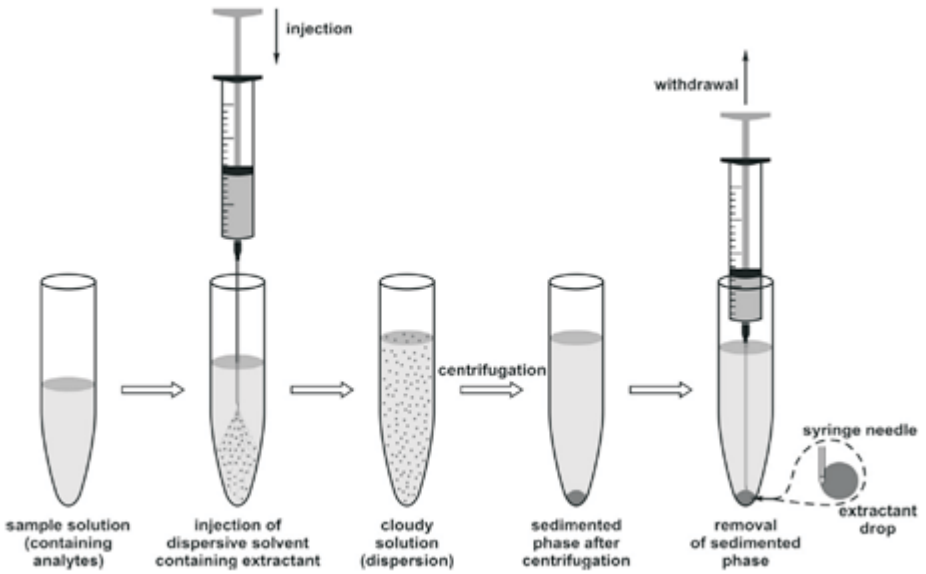


Fig. 1. Scheme of dispersive liquid–liquid microextraction procedure [13].

2. Experimental

2.1 Reagents and chemicals

A standard of 17β-estradiol (Cayman, USA) dissolved in methanol was prepared as stock solution which was thereafter used for preparation of working solutions throughout the experiment. For DLLME, methanol, tetrachloroethane, tetrachloromethane, acetone, chloroform, NaCl, Na_2CO_3, K_2CO_3 were used. For TLC, toluene and ethanol were used as components of mobile phase. 10% solution of phosphomolybdic acid in methanol was used as postchromatographic detection reagent. All chemicals were of analytical grade. Model urine samples for DLLME testing were prepared by adding standard solution of estradiol into urine of a 3-year old boy, thus reaching concentration 0.5 mg/mL. Also, urine sample of a woman in 26th week of pregnancy was used for testing the application of optimized method.

2.2 Instrumentation

DLLME was accomplished using end-capped plastic 5 mL test tubes, 100 μL microsyringe (Hamilton, Switzerland), centrifuge MPW-310 (Poland). For chromatographic analysis, a microsyringe (Hamilton, Switzerland) was used for application of samples (0.3 μL) to the alumina plates with silica gel coating ALUGRAM Sil G/ UV 254 (Macherey-Nagel, Germany) which were thereafter placed into a vertical chromatographic chamber (Lublin, Poland) saturated with vapors of mobile phase, mixture of toluene-ethanol (9:1; v/v). Developed TLC plates were dried in a stream of air at laboratory temperature and then were subjected to postchromatographic detection. First, TLC plates were shortly (1–2 s) dipped into 10% phosphomolybdic acid solution [20] and heated for approximately 10 minutes in oven to 110 °C for specific development of dark blue spots of estrogens on greenish background. Finally, TLC plates were evaluated on densitometer CS-930 (Shimadzu, Japan) at $\lambda = 430$ nm.

3. Results and discussion

Analysis of estrogens is very common due to potential carcinogenic character of these compounds. Because of difficult analysis of estrogens in biological matrices, use of sample-pretreatment methods is crucial for the following analysis, particularly for matrix effect reduction and preconcentration of low amounts of estrogens in such complex samples.

DLLME procedure can be significantly influenced by different factors, such as the volume of extraction and dispersive solvents, extraction time or centrifugation conditions. The selection of the dispersive and extraction solvents is of great importance for the DLLME procedure in order to obtain good extraction. The

requirement for extraction solvent is higher density than water, good extraction capacity for the target compounds and low miscibility in water. On the other hand, high miscibility with dispersive solvent is mandatory. Three halogenated solvents were studied: chloroform (density 1.5 g/mL), tetrachloromethane (density 1.6 g/mL) and tetrachloroethane (density 1.6 g/mL). The dispersive solvent must be miscible in both the aqueous sample and the extraction solvent. Two dispersive solvents were tested with combination with selected extraction solvents: methanol and acetone. Five hundred microlitres of dispersive solvent were mixed with 50 μL of each of extraction solvents before injecting into the sample. These extractions resulted in very poor formation of final organic drop, except for the combination of methanol and tetrachloroethane which was therefore chosen. Various ratios of these two solvents were tested reaching highest efficiency when injecting 500 μL of methanol-tetrachloroethane (9:1; v/v) mixture.

The main problem to deal with during optimization of extraction protocol was the complex character of urine sample resulting in formation of tough precipitation sediment during the final centrifugation thus disabling the removal of organic drop. For solving this problem, addition of salt solution was employed. Three types of salt (K_2CO_3, Na_2CO_3, NaCl) were tested. Only 1 M NaCl significantly improved the progress of extraction. Various extraction times were investigated within the range 30–120 s and it showed that the time does not affect the extraction efficiency significantly. The samples were centrifuged for 5 minutes at 4000 and 10,000 rpm demonstrating better formation of organic drop at 10,000 rpm.

DLLME extracts were analysed by thin-layer chromatography with chemical and densitometric detection. The preliminary overall recovery of DLLME method was 93.1% for model urine sample containing 0.5 mg/mL estradiol. The method provided good linearity within concentration range 0.1–1.0 mg/mL (R^2 = 0.9965) with *LOD* = 0.1 mg/mL. Proposed method was applied to the analysis of free estradiol in real human pregnancy urine (26th week of pregnancy) when 0.3 mg/mL estradiol was determined in DLLME extract (Figure 2).

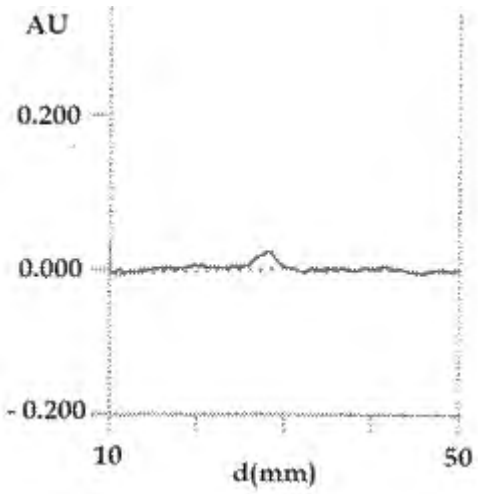


Fig. 2. Densitogram of a real human pregnancy urine (26th week of pregnancy) after dispersive liquid–liquid microextraction. Stationary phase: silica gel; mobile phase: toluene:ethanol (9:1; v/v); sample volume: 0.3 μL; chemical (10% PMA) and densitometric (λ = 430 nm) detection.

4. Conclusions

A preliminary testing of DLLME procedure for isolation of estradiol from urine sample was carried out in this work. Parameters, such as type and volume of extraction and dispersive solvent, salt addition, extraction time and centrifugation conditions were investigated. Optimum conditions were reached after rapid injection of 500 μL methanol-tetrachloroethane (9:1; v/v) mixture to urine sample alkalized with 1 M NaCl to pH ≥ 10. The samples were thereafter centrifuged at 10 000 rpm for 5 minutes. The experiment showed no significant effect of extraction time on the recovery of estradiol. DLLME procedure was evaluated by means of thin-layer chromatography with chemical (10% phosphomolybdic acid) and densitometric (λ = 430 nm) detection. Method provided good linearity within concentration range 0.1–1.0 mg/mL (R^2 = 0.9965) with *LOD* = 0.1 mg/mL. Proposed method was applied to the analysis of free estradiol in real human pregnancy urine when 0.3 mg/mL estradiol was determined in DLLME extract.

Acknowledgments

This work was supported by VVGS-2014-189.

References

[1] Gruber C. J., Tschugguel W., Schneeberger C., Huber J. C.: *N. Engl. J. Med.* **346** (2002), 340–352.
[2] Wend K., Wend P., Krum S. A.: *Front. Endocrinol.* **3** (2012), 1–14.
[3] Simpson E. R.: *J. Steroid Biochem. Mol. Biol.* **86** (2003), 225–230.
[4] D´Ascenzo G., Di Corcia A., Gentili A., Mancini R., Mastropasqua R., Nazzari M., Samperi R.: *Sci. Total Environ.* **302** (2003), 199–209.
[5] Eliassen A. H., Spiegelman D., Xu X., Keefer L. K., Veenstra T. D., Barbieri R. L., Willett W. C., Hankinson S. E., Ziegler R. G.: *Cancer Res.* **72** (2012), 696–706.
[6] Olson S. H., Bandera E. V., Orlow I.: *Am. J. Epidemiol.* **165** (2007), 235–245.
[7] Harkonen P. L., Makela S. I.: *J. Steroid Biochem. Mol. Biol.* **92** (2004), 297–305.
[8] Campbell C. G., Borglin S. E., Green F. B., Grayson A., Wozei E., Stringfellow W. T.: *Chemosphere* **65** (2006), 1265–1280.
[9] Wang S., Huang W., Fang G., He J., Zhang Y.: *Anal. Chim. Acta* **606** (2008), 194–201.
[10] Yan W., Lin J. M.: *Chinese J. Anal. Chem.* **38** (2010), 598–606.
[11] Zou Y., Li Y., Jin H., Tang H., Zou D., Liu M., Yang Y.: *Anal. Biochem.* **421** (2012), 378–384.
[12] Rezaee M., Assadi Y., Hosseini M. R. M., Aghaee E., Ahmadi F., Berijani S.: *J. Chromatogr. A* **1116** (2006), 1–9.
[13] Zgola-Grzeskowiak A., Grzeskowiak T.: *Trends Anal. Chem.* **30** (2011), 1382–1398.
[14] Blair I. A.: *Steroids* **75** (2010), 297–306.
[15] Du X., Wang X., Li Y., Ye F., Dong Q., Huang C.: *Chromatographia* **71** (2010), 405–410.
[16] Hadjmohammadi M. R., Ghoreishi S. S.: *Acta Chim. Slov.* **58** (2011), 765–771.
[17] Chang C. C., Huang S. D.: *Anal. Chim. Acta* **662** (2010), 39–43.
[18] Lima D. L. D., Silva C. P., Otero M., Esteves V. I.: *Talanta* **115** (2013), 980–985.
[19] Wu C. Q., Chen D. Y., Feng Y. S., Deng H. M.: *Anal. Lett.* **45** (2012), 1995–2005.
[20] Reiffova K., Kupcova E.: *J. Planar Chromatogr. – Mod. TLC* **26** (2013), 375–378.

Monitoring of odor nuisance on the adjacent areas to the landfill using Fast GC and sensory analysis

Paulina Lewkowska[a,*], Tomasz Dymerski[a], Jacek Gębicki[b], Jacek Namieśnik[a]

[a] *Gdansk University of Technology, Faculty of Chemistry, Department of Analytical Chemistry, Narutowicza 11/12 Street, 80-233 Gdansk, Poland* ✉ *kolasinska.paulina@wp.pl*
[b] *Gdansk University of Technology, Faculty of Chemistry, Department of Chemical Engineering and Process, Narutowicza 11/12 Street, 80-233 Gdansk, Poland*

Keywords
electronic nose
Fast GC
landfill
LDA
PCA

Abstract
The paper shows the results of investigation on classification of the atmospheric air samples collected in a vicinity of the landfill during August and September period have been presented. The studies was conducted by the use of Fast GC HERACLES II from Alpha MOS company and sensory analysis. About 86% of the atmospheric air samples collected were classified correctly using PCA and LDA. Based on a classification of the atmospheric air samples around the landfill it can be observed that the biggest number of correctly classified samples originated from the directions characterized by odour nuisance. It was the NE direction. The odor intensity of atmospheric air samples from the NE direction was described as 2.5 in VDI 3940 standard and for the other directions was approx. 1.5.

1. Introduction

Municipal waste landfills, belonging to the group of plants generating air pollution, at one time they were placed into the outskirts of cities. Currently, due to the progressive urbanization, the distance between residential areas and landfill is reduced. Unpleasant odors present in ambient air, stimulating the human olfactory receptors and causing odor perception are called odorants. Odors generated from municipal waste landfills are a complex mixture of volatile chemical compounds [1]. The process of municipal waste storage, leading to a reduction of odors emission is often overlooked and odor nuisance of atmospheric air in the area of landfills may negatively impact on the health and comfort of living of society [2]. During the last decade it has witnessed a considerable increase of complaints population living in nearby areas, related to the odors emission occurring in areas located in the vicinity of municipal waste landfills. Therefore, there has been increased interest of the municipal waste landfill impact on the environment [3].

Due to the increasing number of population complaints about air quality occurring in the vicinity of municipal landfills, it is necessary to implement the appropriate procedures to ensure the ability to perform sensory evaluation in order to control emissions [4]. A device which allows for rapid, objective analysis of odor substances is an electronic nose (e-nose) [5–6]. A method in which e-nose is used can be treated as a supplement for method based on field olfactometry [7]. Identification of the individual odoriferous substances or gas odor mixtures is possible by comparing the gaseous sample profiles with reference profiles included in the relevant database. The utilization of electronic nose provides a possibility of monitoring and recording the episodes of odor nuisance from municipal landfill [8].

In this work the research concerning the application of electronic nose based on fast GC and sensory analysis was provided. The subject of the study was atmospheric air samples collected in 2 km range from landfill located Gdansk--Szadolki area. The possibility of the use of an electronic nose device for odor nuisance recognition was investigated.

2. Experimental

2.1 Instrumentation

The measurement set-up consisted of four main elements: HERACLES II device (AlphaMOS, France), air sampling device, the Tedlar bag of 5 dm^3 volume (SKC, USA) and 5 cm^3 syringe (Hamilton, Switzerland). The air sampling device was laboratory-made production (Fig. 1).

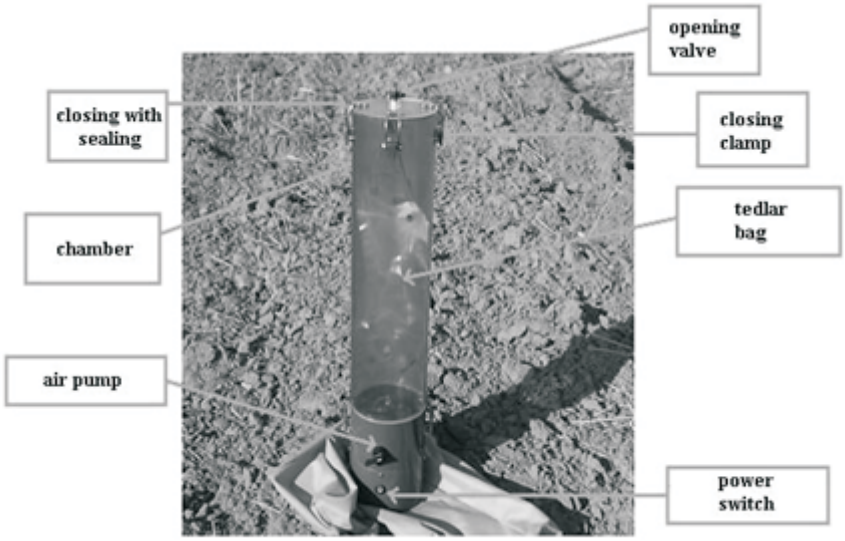


Fig. 1. The view of atmospheric air sampling device.

The measurement procedure consisted in sampling of air directly from the Tedlar bag using the syringe. Then 5 cm^3 air sample was supplied to a proportioner. Sorption of the sample occurred behind the proportioner, inside a sorption trap of Tenax. The analytes were released from the trap after it had been heated to 260°C and the stream was directed to two independent chromatographic--detection systems. The main elements of these systems were two chromatographic columns characterized by different polarity of stationary phase (non-polar MXT-5 and medium-polarity MXT-1701) and two detectors of μFID type. The length of both columns was 10 m. Surface area of chromatographic peaks was utilized in analysis. A single analysis lasted 90 s.

2.2 Procedure of sensory analysis

The tentative studies with the use of water solutions of n-butanol prepared on 5 different level of concentrations, namely 0 ppm v/v, 100 ppm v/v, 200 ppm v/v, 500 ppm v/v, 1000 ppm v/v, was provided for training 4 panelists selected from 20 volunteers. Each of these 4 panelists performed 15 analysis during 5 days for estimation own personal odor threshold. Panelists were selected on the basis of individual repeatability criteria which can be described by the equation:

$$10^S \leq 2.3 \quad (1)$$

where S is standard deviation of individual smell assessment.

In this odor intensity of atmospheric air studies 2 woman and 2 man in age 20–25 have attended. Panelists was trained in terms of proper conducting sensory analysis. They belongs to the group of people who are not smoking cigarettes and with psychophysical condition which was assessed as a very good. One hour before each analysis panelists did not eat and drink. The total of 240 analysis were conducted in 4 localizations in the range of 2 km from the landfill placed in Gdansk Szadolki during 5 days. Each air atmospheric sample was analyzed in 3 replications. The task of each panelist was to assign the odor intensity using verbal odor intensity scale regarding to the instructions included in German VDI 3940 standard. In afore-mentioned standard the intensity of odor should be described as: 0 – without the odor, 1 – very week odor, 2 – week odor, 3 – distinct odor, 4 – strong odor, 5 – very strong odor, 6 – extremely strong odor.

2.3 Research methodology

Investigation of air quality with respect to odour nuisance was performed with the air samples collected within 2-kilometre distance around the landfill along four directions (NE-600, SE-1200, SW-2400 and NW-3000). Localization of the air sampling points around the landfill is illustrated in Fig. 2. The samples were collected during August and September period. Each day 4 samples were collected

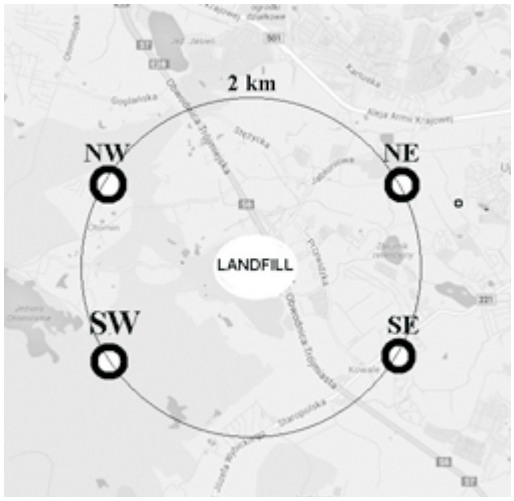


Fig. 2. Map of the municipal landfill with the points of atmospheric air samples collection.

along the aforementioned directions and within the aforementioned distance from the landfill. Selection of the sampling periods was connected with the complaints (on odour nuisance) of the inhabitants of the housing estates neighbouring with the landfill. The atmospheric air samples collected along the other directions (depending on sampling period) were treated as the reference samples as no complaints on odour nuisance were acknowledged in these directions. There was no precipitation during the sampling periods. The samples were collected into the Tedlar bags of 5 dm^3 volume using a device called lung sampler, which was designed and built in the Department of Analytical Chemistry of the Gdansk University of Technology. The total of 60 atmospheric air samples were collected around the landfill (4 collection points times 5 days times 3 repetitions).

Classification of the atmospheric air samples with respect to the sampling points (thus different odour nuisance) utilized the linear discriminant analysis (LDA) method.

Principal component analysis was utilized for visual evaluation of an ability of discrimination between the air samples originating from the places with odour nuisance and the places without it. Classification of the atmospheric air samples with respect to their odour nuisance was performed using LDA employing free R software being a part of Free Software Foundation (Free Software Foundation, USA).

3. Results and discussion

Fig. 3 presents the PCA results for odour intensity of the atmospheric air samples collected in a vicinity of the landfill during August and September period. Also in this case the two-dimensional plane reveals two distinct point clusters attributed to the places of samples collection. Fig. 3 shows that one of the clusters corresponds to the samples collected from the NE direction, where odour

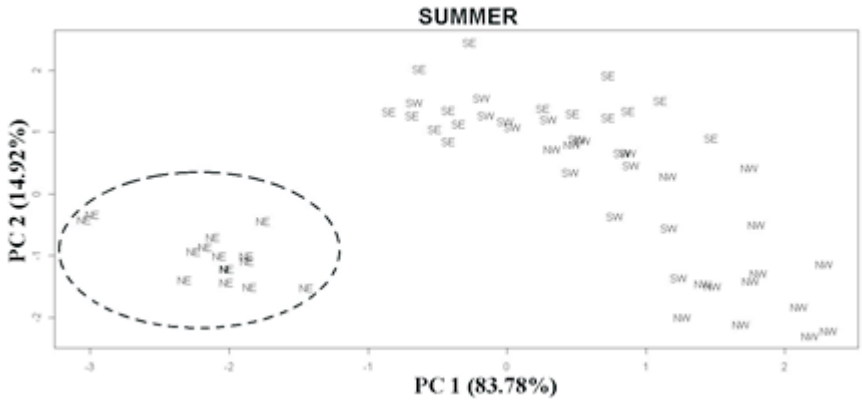


Fig. 3. PCA result for odour intensity of the atmospheric air samples collected during summer season from four directions localized in a vicinity of the municipal landfill.

nuisance complaints were recorded during that season. The second cluster represents the remaining directions for odour intensity of the atmospheric air samples collection. This region corresponds to the air samples collected from the places, where no complaints on odour nuisance were recorded.

Table 1 presents a matrix of correctly classified odour intensity of atmospheric air samples collected in a vicinity of the landfill during summer season. The classification was performed with the cross-validation supported LDA. The 86.7% of the samples from the selected directions were classified correctly. The biggest number of correctly classified samples was equal to 15 and originated from the NE direction. In the remaining cases the following number of samples were classified correctly: 13 from the NW direction, 12 from the SE as well as from the SW direction. As far as the NE direction is concerned, characterized by odour nuisance complaints, correct classification amounted 100%.

In the case of LDA classification performed exclusively on the training set (without cross-validation method) the correct classification level was 95.0%.

Table 1
Cross-validation supported LDA classification of odour intensity of the atmospheric air samples collected in a vicinity of the municipal landfill during summer season. Measurement data for classification were obtained with the electronic nose HERACLES II.

Cross-validation method: 86.7%				
	NE	SE	SW	NW
NE	15	0	0	0
SE	1	12	2	0
SW	0	1	12	2
NW	0	1	1	13

Table 2
The odor intensity of atmospheric air samples collected in the 2 km distance from landfill located in Gdansk Szadolki

Direction	Odor intensity
NE	2.5±0.4
SE	1.5±0.3
SW	1.4±0.3
NW	1.5±0.3

In Table 2 the average geometric odor intensities of the atmospheric air samples collected around the landfill located in Gdansk-Szadolki are were presented. It can be observed in direction NE the values of the odor intensity of air atmospheric samples were higher and statistically different on the established level of significance ($\alpha = 0.05$) in comparison to the other samples.

4. Conclusions

The aim of investigation was discrimination and classification of the atmospheric air samples collected in four directions, along which pollutants are spread around the municipal landfill. The studies were oriented towards the ability of discrimination of the air samples containing odorants. Commercial electronic nose instrument HERACLES II of Fast GC type by Alpha MOS was utilized in the analysis and sensory panel consisted 4 person.

Based on a classification of the atmospheric air samples around the landfill it can be observed that the biggest number of correctly classified samples originated from the direction characterized by odour nuisance. It was the NE direction during August and September period. It can be also observed sensory panel proved the odor intensity of atmospheric air samples was the highest for this direction. The results of this research will be utilized by the authors during elaboration of a stationary version of the electronic nose instrument for continuous monitoring of atmospheric air with respect to odour nuisance.

Acknowledgments

The research was supported by the Grant No. PBSII/B9/24/2013 from the National Centre for Research and Development.

References

[1] Gallego E., Perales J.F., Roca F.J., Guardino X.: *Sci. Total Environ.* **470–471** (2014), 587–599.
[2] Lewkowska P., Dymerski T., Namieśnik J.: *Crit. Rev. Env. Sci. Tec.* in press, DOI: 10.1080/10643389.2015.1010429.
[3] Kim H., Lee, H., Choi E., Choi I., Shin T., Im H., Ahn S.: *Chemosphere* **117** (2014), 394–401.
[4] Wardencki W., Biernacka P., Chmiel T., Dymerski T.: *Proc. ECOpole.* **3** (2009), 273–279.
[5] Dymerski T., Gębicki J., Wardencki W., Namieśnik J.: *Sensors* **13** (2013), 15954–15967.
[6] Dymerski T., Gębicki J., Wardencki W., Namieśnik J.: *Sensors* **14** (2014), 10709–10724.
[7] Gębicki J., Dymerski T., Rutkowski S.: *Environ. Prot. Eng.* **40** (2014), 103–116.
[8] Gębicki J., Dymerski T., Namieśnik J.: *Chem. Eng. Trans.* **40** (2014), 85–90.

Determination of arsenic by UV-photochemical generation of its volatile specie with AAS detection

JANA SMOLEJOVA, ANNA VLCKOVA, ONDREJ LINHART*, VACLAV CERVENY, JAKUB HRANICEK, PETR RYCHLOVSKY

Charles University in Prague, Faculty of Science, Department of Analytical Chemistry, Hlavova 8, 128 43 Prague 2, Czech Republic ✉ *ondrej.linhart@natur.cuni.cz*

Keywords
AAS
arsenic
flow injection analysis
generation of volatile compounds
interference study

Abstract
The work was focused on the development of analytical method for determination of arsenic in water sample using of its volatile compounds generation. The method involves the combination of flow injection analysis, UV-photochemical generation of volatile compounds of arsenic and atomic absorption. Attained absorbance was very low after the optimization. In next step, the influence of selected interferents on UV-photochemical generation was investigated with the aim to find a suitable reaction modifier which would improve sensitivity of arsenic determination. Bi^{3+} was found as the best reaction modifier for its persisting effect. The activation with concentration of 10 mg L^{-1} of Bi^{3+} increases the absorbance of arsenic approximately eleven times compared to signals without activation. The limit of detection achieved was 18 μg L^{-1}, sensitivity was 1.144×10^{-3} L μg^{-1}, repeatability was 4.5 % (relative standard deviation) and linear dynamic range was 60–500 μg L^{-1}.

1. Introduction

UV-photochemical generation is one of the possible ways of the generation of volatile species of arsenic [1, 2]. It is based on anaerobic photolytic decomposition of aliphatic organic acids with low molecular weight. Hydrocarbons, their radicals and carbon dioxide are products of this reaction as it is described by equation [3]:

$$R{-}COOH \xrightarrow{h\nu} R^{\cdot} + {}^{\cdot}COOH \longrightarrow RH + CO_2 \quad (1)$$
$$(R = C_nH_{2n+1},\ n = 0, 1, 2)$$

Hydrocarbon radicals are taken up by trivalent arsenic to form stable substituted compounds as shown in equation

$$3\,R{-}COOH + H_3AsO_3 \xrightarrow{h\nu} 3\,CO_2 + R_3As + 3\,H_2O \quad (2)$$
$$(R = C_nH_{2n+1},\ n = 0, 1, 2)$$

For spontaneous release of the compounds generated element from a solution it is necessary that the products formed are sufficiently volatile for spontaneous release of the compounds generated from a solution. Such compounds containing the determined element are formed by photolysis of formic acid, acetic acid and propionic acid [4].

The composition of volatile compounds formed by UV-photochemical generation depends on the type of acid which is providing radicals. The most volatile compounds can be produced in presence of formic acid. Due to the reaction times published [1] as well as due to the volatility of the arsenic compounds produced [5], formic acid was chosen as the most suitable reaction medium for experiments carried out.

The lack of literature data about the determination of arsenic when using UV-photochemical generation of its volatile compounds was the motivation of this work. In contrary, there is a lot of information about determination of other hydride forming elements [6] like selenium [7, 8], antimony, bismuth and even transition metals [9–12] in published articles.

2. Experimental

2.1 Reagents and chemicals

Standard solution of As^{+III} was prepared by weighting of As_2O_3 (> 99.5 %, Sigma-Aldrich), dissolved in deionized water with KOH (89.0 %, Lach-Ner, Czech Republic). Standard solution of 0.75 mol L^{-1} HCOOH (≥ 98%, Sigma-Aldrich) was prepared fresh daily. Solution of interferents were prepared from stocking solutions of Fe^{3+} (1000±2 mg L^{-1}, Merck), Co^{2+} (1000±2 mg L^{-1}, Merck), Ni^{2+} (1000±5 mg L^{-1}, Analytika, Czech Republic), Cu^{2+} (1000±5 mg L^{-1}, Analytika, Czech Republic), Se^{4+} (1000±2 mg L^{-1}, Analytika, Czech republic), Bi^{3+} (1000±5 mg L^{-1}, Merck), SO_4^{2-} (1000±0.002 mg L^{-1}, Merck), Cl^- (1000±0.002 mg L^{-1}, Merck), HNO_3 (≥ 65 %, Merck), ethanol (≥ 99.5 %, Merck), mercaptoethanol (≥ 99.0 %, Sigma-Aldrich), triethanolamine (≥ 98 %, Sigma-Aldrich), acetonitrile (≥ 99.8 %, Sigma-Aldrich) or weighting of L-cysteine hydrochloride monohydrate (≥ 98 %, Sigma-Aldrich), TiO_2 (≥ 99.5%, size nanoparticles ~ 21 nm, Aldrich). The solutions were diluted with deionized water (18 MΩ cm) MilliQPLUS (Millipore, USA).

2.2 Instrumentation

- Atomic absorption spectrometer Varian SpectrAA-300A (Varian, AUS)
- Programmable peristaltic pump Masterflex (Cole-Parmer, USA)
- Power supply for the UV-generator Modus SB 18 (Modus s.r.o., CZE)
- As-hollow cathode lamp (Heraeus, DEU), wavelength 193.7 nm, spectral interval 0.5 nm, supply current 10 mA.

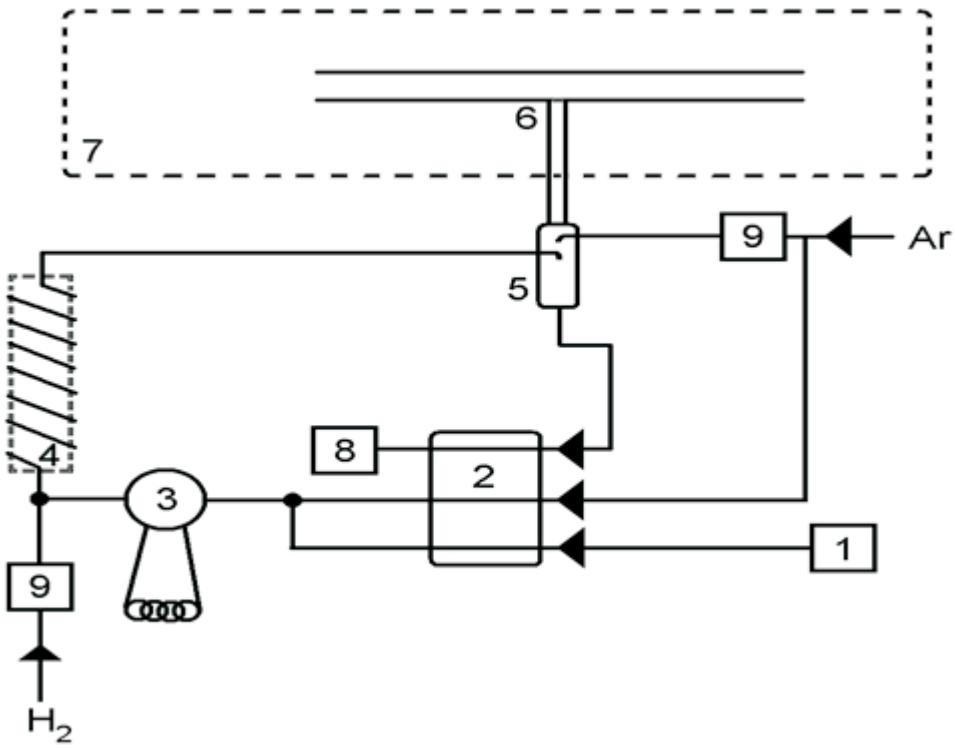


Fig. 1. The instrumental set-up: (1) reservoir bottle with solution of HCOOH, (2) peristaltic pump, (3) six-way injection valve, (4) reaction coil with UV-generator, (5) gas-liquid separator, (6) atomizer, (7) AAS, (8) waste bottle, (9) gas flow controller.

- Laboratory made quartz atomizer with integrated gas-liquid separator which was externally heated to 950 °C by EHA 10 (RMI, CZE)
- Low-pressure Hg UV lamp (253.7 nm, 20 W, Ushio, JPN)
- Connecting Tygon tubing of different sizes (Cole-Parmer, USA)
- PTFE tubing of different ID (Supelco, USA)
- Connecting material (Supelco, USA)
- Mass flow controller (Cole-Parmer, USA)
- Six-way injection valve FIA V-451 (Upchurch Scientific, USA)
- Syringe size 10 ml (Hamilton, USA)
- Analytical balance RC 210D (Sartorius, USA)

The instrumental set-up which was used for this analytical method is depicted in Fig. 1.

3. Results and discussion

3.1 Optimization of conditions for UV-photochemical generation

Firstly, it was necessary to find optimum condition for UV-photochemical generation of volatile arsenic compounds. Following key parameters were optimized: the flow rate of the carrier solution, HCOOH concentration in this solution, the carrier gas (Ar) and the reaction gas (H_2) flow rates, reaction coil length, the sampling loop volume and atomization temperature. The optimum experimental conditions were found to achieve a sufficient signals as well as the highest possible efficiency of the generation of volatile arsenic compounds.

The presence of hydrogen is necessary for successful UV-photochemical generation of volatile compound of arsenic and for its detection by atomic absorption. According to the dependence shown in Fig. 2, almost no volatile product containing arsenic was formed without hydrogen addition prior the UV-photoreactor. This fact was proved by selection of various placements of hydrogen inlet. 30 mL min^{-1} of hydrogen was selected as the optimum hydrogen flow rate because

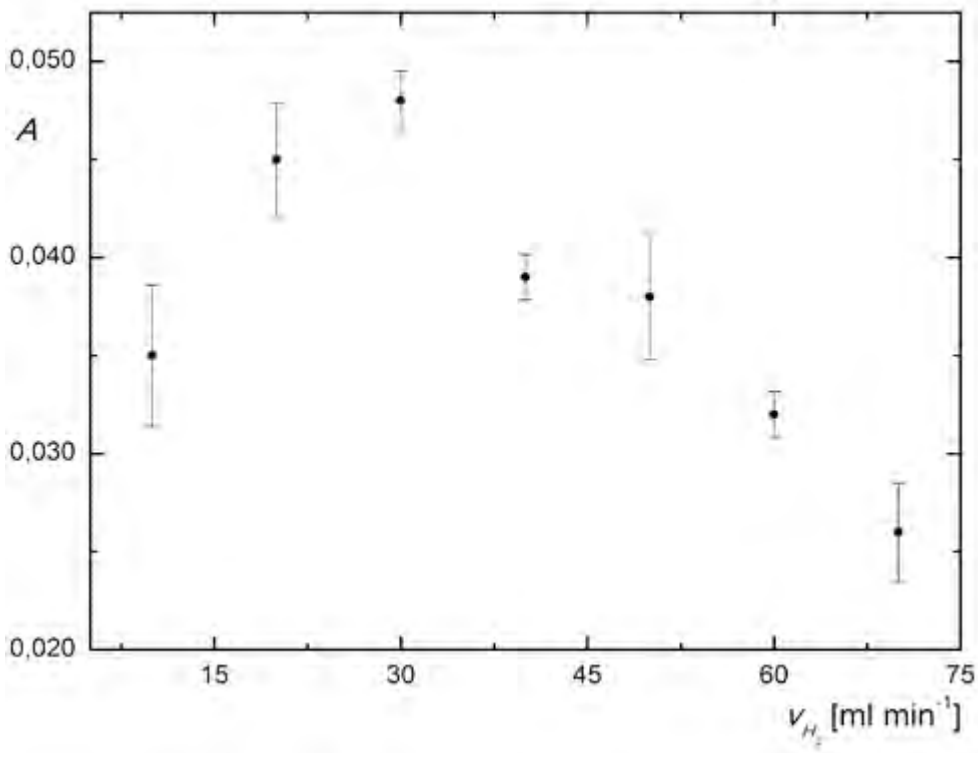


Fig. 2. Dependence of absorbance on reaction gas flow rate ($c(As^{+III})$ = 0.4 mg L^{-1}, c(HCOOH) = = 1.5 mol dm^{-3}, v(Ar) = 24 m^L min^{-1}, v(HCOOH) = 2 m^L min^{-1}, V_{DS} = 600 µL, L = 251 cm, t = 950 °C).

Table 1
Optimum condition.

Parameter	Value
HCOOH concentration	0.75 mol L^{-1}
H_2 flow rate	30 mL min^{-1}
A_2 flow rate	24 mL min^{-1}
Sampling loop volume	600 μL
Carrier flow rate	2.0 mL min^{-1}
Reaction coil length	251 cm
Atomization temperature	950 °C

of the highest response recorded. More hydrogen inflow resulted in signal depression. The cause is probably in the shortening of stay of the analyte in the UV-photoreactor.

There were used two ways of input of inert gas into the instrumental set-up. The aim of the first inlet prior the six-way injection valve was to segment the flow of the carrier solution and to prevent broadening of the zone of injected sample. The highest absorbance was obtained for tubes with an inner diameter of 0.51 mm which corresponded to 0.33 mL min^{-1} of Ar for the same rotation used for pumping of the carrier solution. Secondly, argon was introduced into the gas-liquid separator. Its presence was necessary for release of the arsenic volatile compound from the liquid there and for its transport to the atomizer. The best analytical signal was provided by 24 mL min^{-1} of carrier gas (Ar).

HCOOH is the most important component of the carrier solution. Therefore, its concentration is logically the key parameter to be optimized. The maximum signal was achieved at 0.75mol L^{-1} in the dependence of arsenic absorbance on formic acid concentration and it was surprisingly narrow.

The summary of optimum conditions is reported in Tab. 1.

3.2 Interferences

The interferents were divided into three groups. Negative interferences occured in presence of nickel(II) ions, copper(II) ions, chloride ions and mercaptoethanol. Minimally interfering agents are nitric acid, ferric ions, ethanol, sulfate ions, titanium dioxide, and L-cysteine. The presence of cobalt ions, acetonitrile, triethanol amine, selenite ions and bismuth(III) ions increased absorbance of arsenic. Bismuth(III) ions at a concentration of 10 mg L^{-1} increased the absorbance significantly. The results of further experiments showed that the effect of the activation by bismuth(III) ions persisted for all the measurements done at the same day. The mechanism of the inner modification of the apparatus will be the subject of future investigation.

3.3 Calibrations

Consequently, calibration dependences were measured and figures of merit of the proposed analytical method were found under the optimum conditions. Sensitivity (expressed as the slope of the calibration line) was determined as the main character, then the limit of detection and quantification, repeatability and linear dynamic range were calculated on the base of results attained.

Fig. 3 shows two calibration curves, without and after the activation by bismuth(III) ion respectively. A comparison of figures of merit summarized is presented in Tab. 2. The limits of detection and quantification moved to the lower concentration level as well as linear dynamic range after the activation. On the other hand, LDR became shorter in this case. From equations of the calibration lines, the signal enhancement factor (calculated as a ratio of both sensitivities) was calculated. Its value is 10.8.

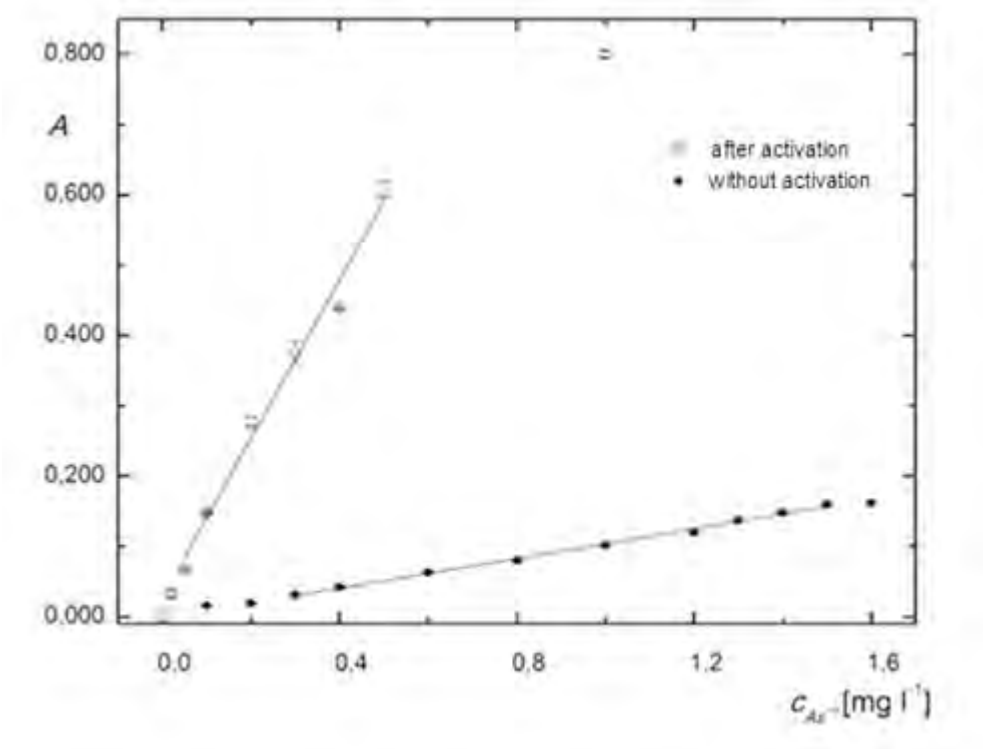


Fig. 3. Calibrations (c(HCOOH) = 0.75 mol dm^{-3}, v(H_2) = 30 mL min^{-1}, v(Ar) = 24 mL min^{-1}, v(HCOOH) = 2 mL min^{-1}, V_{DS} = 600 µL, L = 251 cm, t = 950 °C).

Table 2
Figures of merit.

	Without activation	After activation
LOD [μg L^{-1}]	89	18
LOQ [μg L^{-1}]	300	60
Sensitivity [L μg^{-1}]	1.059×10^{-4}	1.144×10^{-3}
Repeatability [RSD %]	1.9	4.5

4. Conclusions

An analytical method for determination of arsenic using its UV-photochemical volatile compound generation was developed in the presented work. All the measurements were realized in FIA arrangement. Atomic absorption with externally heated quartz tube atomizer was used for detection of arsenic.

Followed the optimization, a suitable reaction modifier was found within interference study. Bismuth(III) ions at concentration 10 mg L^{-1} significantly increased the absorbance of arsenic almost eleven times and its effect persisted for the rest of the day.

The proposed method is distinguished by a detection limit of 18 μg L^{-1} of arsenic, by a sensitivity of 1.144×10^{-3} L μg^{-1}, by a repeatability of 4.5 % and by a linear dynamic range 60–500 μg L^{-1} under the optimum conditions and after the activation by bismuth(III).

Acknowledgments

This work was financially supported by the Charles University in Prague (Projects UNCE204025/-2012, GAUK152214 and SVV260205).

References

[1] Sturgeon R.E., Willie S.N., Mester Z.: *J. Anal. Atom. Spectrom.* **21** (2006), 263–265.
[2] Guo X., Sturgeon R.E., Mester Z., Gardner G.J.: *Anal. Chem.* **76** (2004), 2401–2405.
[3] Guo X., Sturgeon R.E., Mester Z., Gardner G.J.: *J. Anal. Atom. Spectrom.* **20** (2005), 702–708.
[4] McSheehy S., Guo X., Sturgeon R.E., Mester Z.: *J. Anal. Atom. Spectrom.* **20** (2005),709–716.
[5] Sturgeon R.E., Willie S.N., Mester Z.: *J. Anal. Atom. Spectrom.* **21** (2006), 263–265.
[6] Zheng Ch., Ma Q., Wu L., Hou X., Sturgeon R.E.: *Microchem. J.* **95** (2010), 32–37.
[7] Suzuki T., Sturgeon R.E., Zheng Ch., Hioki A., Nakazato T., Tao H.: *Anal. Sci.* **28** (2012), 807–811.
[8] Zheng Ch., Wu L., Ma Q., Lv Y., Hou X.: *J. Anal. Atom. Spectrom.* **23** (2008) 514–520.
[9] Nóbrega J.A., Sturgeon R.E., Grinberg P., Gardner G.J., Brophy Ch.S., Garcia E.E.: *J. Anal. Atom. Spectrom.* **26** (2011), 2519–2523.
[10] Deng H., Zheng Ch., Liu L., Wu L., Hou X., Lv Y.: *Microchem. J.* **96** (2010), 277–282.
[11] Zheng Ch., Sturgeon R.E., Brophy Ch.S., He Sh., Hou X.: *Anal. Chem.* **82** (2010), 2996–3001.
[12] Zheng Ch., Sturgeon R.E., Hou X.: *J. Anal. Atom. Spectrom.* **24** (2009), 1452–1458.

Determination of selected components of human urine by electrophoresis in a short capillary with pressure-assisted sampling

Anna Makrlíková[a,*], František Opekar[a], Petr Tůma[b]

[a] *Charles University in Prague, Faculty of Science, Department of Analytical Chemistry, Hlavova 2030/8, 128 43 Prague 2, Czech Republic* ✉ *anna.makrlikova@natur.cuni.cz*
[b] *Charles University in Prague, Third Faculty of Medicine, Institute of Biochemistry, Cell and Molecular Biology, Ruská 2411/87, 100 00 Prague 10, Czech Republic*

Keywords
dual detection
human urine
hydrodynamic injection
short-capillary electrophoresis

Abstract
A hydrodynamic sample introduction method has been proposed for electrophoresis in short capillary. The separation electrolyte flushes sample from the loop of a six-way sampling valve and carries it to the injection end of the capillary. At the time when the sample zone reaches the capillary, a short pressure impulse is generated in the electrolyte stream, which provides injection of the sample into the capillary. Then the electrolyte flow is stopped and the separation voltage is turned on. The method was used for the determination of selected components in urine. The determination was performed in a capillary with an overall length of 10.5 cm and two base electrolytes was tested. A, dual contactless conductivity and UV spectrometric, detection was used.

1. Introduction

Capillary electrophoresis is an ideal technique for the separation of complicated clinical samples because of its high separation efficiency, short separation time and minimal requirements on the amounts of samples and reagents. Commercially available capillary electrophoresis apparatuses contain long capillaries (several tens of cm) and the separation time is usually 5–30 minutes [1]. It is desirable that the separation time be much shorter, affecting the number of samples that can be analyzed in unit time and thus the price of the analysis.

Shortening of the separation pathway is an effective technique for increasing the rate of separation. One of the options involves performance of electrophoresis on a glass or plastic microchip [2]. This electrophoresis requires using a special separation system – an electrophoretic chip. More preferred is the use of standard electrophoretic capillaries. A technically simple shortening of the separation pathway is short-end injection, where the sample is injected into a site close to the detector, commercial instrumentation can frequently be used for this purpose [3]. Greater experimental variability can be attained using special apparatuses for

separation directly in short capillaries with overall length of about 10 cm. A basic experimental problem when using separation in short capillaries lies in their limited mobility. A short capillary cannot be handled in the same way as the capillaries used in standard electrophoretic systems. It has to be designed so that all the necessary steps can be performed without necessity to move the capillary.

Injection of the sample into a separation capillary is generally an important point in achieving high-performance electrophoretic separation. An easily available option lies in sample injection based on the principle described in the work [4]. The injection end of the capillary is loosely inserted into a tube through which the background electrolyte is flowing during the injection, into which the sample is injected from the loop of a six-way valve. The sample can be injected into the capillary during the time that the sample zone flows around the injection end of the capillary. However, in electrokinetic sample injection, the amount of injected substance in the sample depends on its electrophoretic mobility, on total conductivity and composition of the sample matrix. The problems can be solved by the use of pressure-assisted injection described in this work. This hydrodynamic sampling controlled by pressure pulse can be used for determination of ammonia, histidine, creatinine, uric and hippuric acid in human urine.

2. Experimental

2.1 Reagents and chemicals

To illustrate the practical applicability of the proposed pressure-assisted injection, some of the components in human urine were determined. Urine samples were obtained from a healthy volunteer and analyzed on the day of sampling. Urine was only filtered using a single-use injection-needle PVDF filter and then diluted 50× or 100× with deinonized water. Two base electolytes were tested 50 mM MES + 5 mM NaOH (pH = 5.10) and 1 M acetic acid + 1.5 mM crown ether 18-crown-6 (pH = 2.40).

2.2 Instrumentation

Two detection systems, laboratory-made C^4D (450 kHz/17 V) and UV (214 nm), were tested.

The injection end of the electrophoretic apparatus, Fig. 1A, consists of plastic components used for connecting tubes of small dimensions (1, 2). The electrophoretic ground electrode (3) is a 1 cm long piece of stainless steel tube, plastic components 1 and 2 fix the electrode in a stable position. The injection end of the capillary is inserted into another PTFE tube (5), which the background electrolyte solutions with the sample flows during the injection. The shut-off valve (10) closes the exit PTFE tube (11), this creates a pressure pulse in the apparatus and sample is injected into the capillary. The hydrodynamic resistance (13) causes a sharper increase in the pressure when the shut-off valve is closed.

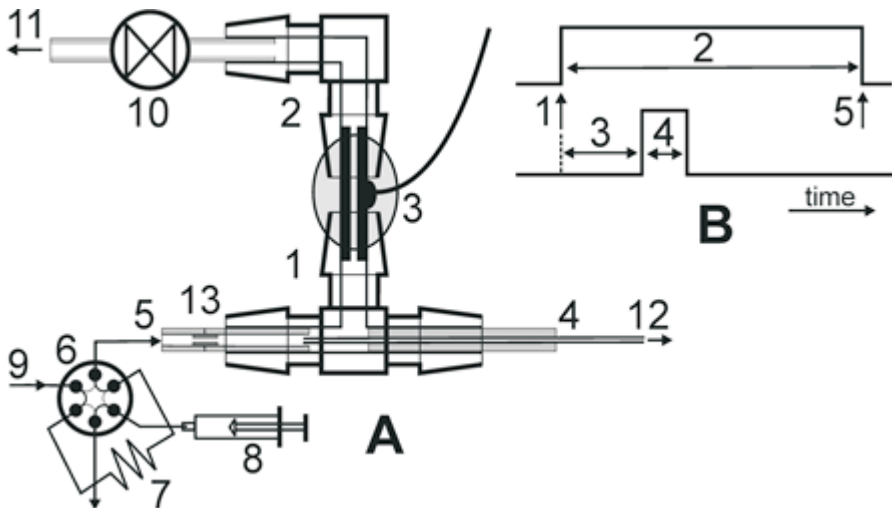


Fig. 1. Electrophoretic apparatus; scheme of the injection part (A) and scheme of the time dependence of the injection sequence (B).

(A) 1, 2 – barbed tubing fittings; 3 – stainless steel tube (ground electrode of the high voltage source) with soldered connection, insulated with hot melt adhesive; 4 – separation capillary inserted in PTFE tube; 5 – PTFE tube; 6 – six-way sampling valve; 7 – injection loop with volume of 40 μl; 8 – filling the injection loop; 9 – background electrolyte feed from the stock reservoir pumped by a linear pump; 10 – shut-off valve; 11 – waste; 12 – to the detector and end vessel, 13 – piece of capillary creates a hydrodynamic resistance.

(B) 1 – initiation of injection; 2 – time of operation of the linear pump; 3 – delay of the shut-off valve; 4 – time of activation of the shut-off valve, i.e. time of sample injection into the capillary; 5 – turning on the high voltage source and commencement of the separation.

Fig. 1B depicts the time sequence of the injection. The injection time is commenced by turning on the linear pump for the background electrolyte and simultaneously switching the six-way valve from the "load" position (filling the injecting loop) to the "inject" position. At the time when the sample zone in the background electrolyte reach the injection end of the capillary, the shut-off valve is activated for a pre-defined time and pressure pulse injects the sample into capillary. The linear pump is turned on for long enough time for the sample zone to reach a sufficient distance from the capillary. After turning off the pump, high voltage is turned on and the separation is commenced.

The separation was performed in a standard fused silica capillary, 50 μm *i.d.*, 363 μm *o.d.*, with total length 10.5 cm and length to the detector 8 cm. The electrophoretic apparatus was laboratory-made and it is described in a previous publication [5]. The separation capillary leads from the injection part to the dual detector and to the end vessel with the high-voltage electrode of the high voltage source (5 kV). In subsequent measurements were used optimized parameters: time of activation of the linear pump (2) 10 s, background electrolyte flow-rate 1 ml/min, time of delayed activation of the shut-off valve (3) 5 s and time of activation of the shut-off valve (4) 0,5 s.

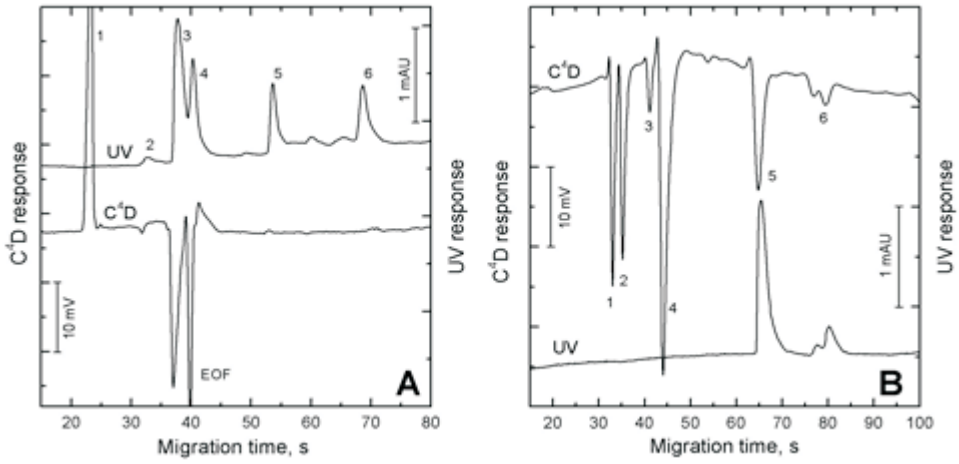


Fig. 2. Part of the electropherograms of human urine recorded by C^4D and the UV detector.
(A) Urine diluted with water 1:50, background electrolyte 50 mM MES + 5 mM NaOH (pH = 5.10), separation voltage/current 5 kV/3 μA. Identification of peaks: 1 – unseparated inorganic ions, 2 – histidine, 3 – creatinine, 4 – neutral substances absorbing UV radiation, 5 – uric acid, 6 – hippuric acid.
(B) Urine diluted with water 1:100, background electrolyte 1 M acetic acid + crown ether 18-crown-6 (pH = 2.40), separation voltage/current 5 kV/13 μA. Identification of peaks: 1 – NH_4^+ , 2 – K^+, 3 – Ca^{2+}, 4 – Na^+, 5 – creatinine, 6 – histidine.

3. Results and discussion

The determination of important biochemical analytes in human urine was selected for verification of the practical applicability of the tested injection method. Separation of these substances was first tested in background electrolyte with composition 50 mM MES + 5 mM NaOH (pH = 5.10), see Fig. 2A. Before EOF on the C^4D recording positive peak of the mixed zone of inorganic ions can be seen, followed by the negative peaks of the organic cations histidine and creatinine. In the UV recording EOF is followed by the separated peaks of uric and hippuric acids. Acidic BGE with composition 1 M acidic acid + crown ether 18-crown-6 (pH = 2.40), Fig. 2B, was used for the separation cations in urine, because addition of 18-crown-6, which complexes K^+, allows separation NH_4^+ and K^+ ions.

The obtained separation efficiencies commonly attain values of about 100,000 theoretical plates/m, these values are fully comparable with the separation efficiencies for classical hydrodynamic injection of samples connected with exchange of vials. The results of the determination of the monitored components in urine are given in Tables 1 and 2. The standard addition method was used and the results are the median of three determinations. Table 1 also gives the range of concentrations in which this components can be present in human urine [6].

Table 1
Determination of creatinine, histidine, uric and hippuric acid (detection method in parentheses). Sample: human urine diluted with water 1:50, background electrolyte: 50 mM MES + 5 mM NaOH (pH = 5.10), separation voltage/current: 5 kV/3 μA.

Analyte	Migration time [s]	Concentration [mg/l]	RSD [%]	Range [6] [mg/l]	N^a [1/m]
Histidine (C^4D)	32±1	67±7	5.1	40–330	136 200
Histidine (UV)		61±6	4.6		–
Creatinine (C^4D)	36±1	1031±233	10.2	670–2150	52 300
Creatinine (UV)		1209±364	13.7		24 900
Uric acid (UV)	54±3	392±20	2.3	40–670	118 000
Hippuric acid (UV)	69±5	539±33	2.7	50–1670	150 000

[a] The number of theoretical plates was calculated from the formula $N = 5.54(t_M/w_{1/2})^2$, where t_M is the migration time and $w_{1/2}$ is the peak width at half-height.

Table 2
Determination of ammonia ions and creatinine (detection method in parentheses). Sample: human urine diluted with water 1:100, background electrolyte: 1 M acetic acid + 1.5 mM crown ether 18-crown-6 (pH = 2.40), separation voltage/current 5 kV/13 μA.

Analyte	Migration time [s]	Concentration [mg/l]	RSD [%]	N [1/m]
NH_4^+	34±0	515±95	8.3	132 000
Creatinine (C^4D)	68±0	1666±75	2.0	95 600
Creatinine (UV)		1717±159	4.2	62 700

4. Conclusions

As an alternative to electrokinetic injection, a hydrodynamic sample introduction method controlled by pressure pulse has been proposed for short-capillary electrophoresis. Injection was tested by determining several components of human urine. Separation has the general advantage of speed, but two different separation buffers had to be used. Creatinine can be determined in both background electrolytes, the determination in MES-NaOH background electrolyte is less accurate, because creatinine peak is immediately next to the EOF peak, complicating its evaluation. In acetic background electrolyte histidine is poorly separated from the other components of the urine, probably from its methyl derivatives.

Acknowledgments

This work was financially supported by the Charles University in Prague, project SVV 260205, project PRVOUK 31 and by the Grant Agency of the Czech Republic, project 15-03139S.

References

[1] Opekar F., Coufal P., Štulík K.: *Chem. Rev.* **109** (2009), 4487–4499.
[2] Shang F.J., Guihen E., Glennon J.D.: *Electrophoresis* **33** (2012), 105–116.
[3] Glatz Z.: *Electrophoresis* **34** (2013), 631–642.
[4] Tůma P., Opekar F., Jelínek I.: *J. Chromatogr. A* **883** (2000), 223–230.
[5] Vochyánová B., Opekar F., Tůma P.: *Electrophoresis* **35** (2014), 1660–1665.
[6] Putnam D. F.: Report No. NASA CR-1802, Composition and concentrative properties of human urine, McDonnell Douglas Astronautic company, Huntington Beach, California, <http://ntrs.nasa.gov/archive/nasa/casi.ntrs.nasa.gov/19710023044.pdf>, accessed 28th June, 2015.

Efficient generation of arsanes coupled with HPLC or with cryotrapping for speciation analysis of arsenic

Karel Marschner[a, b, *], Stanislav Musil[a], Petr Rychlovský[b], Jiří Dědina[a]

[a] *Institute of Analytical Chemistry of the CAS, v. v. i., Veveří 97, 602 00 Brno, Czech Republic* ✉ *karel.marschner@biomed.cas.cz*
[b] *Charles University in Prague, Faculty of Science, Department of Analytical Chemistry, Hlavova 8, 128 43 Prague, Czech Republic*

Keywords
arsenic
cryotrapping
HPLC
hydride generation
speciation analysis

Abstract
The aim of this work was to find conditions of hydride generation to generate four arsenic species (arsenite, arsenate, monomethylarsonate, dimethylarsinate) with the same efficiency in a flow injection and batch mode. In the next step such generators and optimized conditions of hydride generation were employed for speciation analysis of arsenic. In the first case arsenic species were separated by HPLC and the sample injection valve was substituted with the HPLC outlet. In the second case the batch generator was connected with a cryotrapp that separated generated arsanes according to their different boiling points. Due to same generation efficiency (and thus sensitivity) single species standardization could be used in the both approaches. This has been successfully demonstrated on urine sample with postcolumn hydride generation so far. The preliminary results with a cryotrapp are also very promising.

1. Introduction

Arsenic is a known toxic metalloid. Its toxicity strongly depends on its chemical form, therefore it is desirable to know speciation of arsenic in the sample. The most toxic arsenic species are arsenite (iAs^{III}) and arsenate (iAs^{V}). Monomethylarsonate (MMA^{V}) and dimethylarsinate (DMA^{V}) exhibit lower toxicity and other organically bound arsenic species are even non-toxic. By coincidence the toxic arsenic species are "hydride active", i.e. they can be converted to their corresponding hydrides (arsanes) by the reaction with $NaBH_4$ [1]. This can be utilized for speciation analysis. Actually, there are three possibilities to employ hydride generation (HG) for speciation analysis [2]: selective HG, generation of substituted hydrides and postseparation HG.

Selective HG "separates" arsenic species by means of different conditions of HG [3]. The second approach is generation of substituted hydrides, when the resulting gaseous mixture of alkylsubstituted arsanes is separated, usually by

cryotrapping [4] or gas chromatography. The last approach is postseparation HG, most commonly after HPLC, that is used to increase sensitivity and improve specificity. The advantage of the HG step lies in the separation of analyte from the liquid matrix and in much higher introduction efficiency (up to 100%) to the detector in comparison to nebulization techniques.

100% efficiency of HG from all As "hydride active" species is desirable. However, this is hardly achieved without pre-reduction of iAs^{V}, MMA^{V} and DMA^{V} to their trivalent forms. The pre-reduction can lead to contamination of a sample or, if it is performed on-line, more complicated generators must be used [5].

The aim of this work was to (i) develop an efficient HG from iAs^{III}, iAs^{V}, MMA^{V} and DMA^{V} in both batch and flow injection (FI) mode using only HCl medium and $NaBH_4$, (ii) connect such HG to HPLC and a cryotrapp (CT) and (iii) apply the developed methods to real (human urine) samples. The in-house designed atomic fluorescence spectrometer (AFS) was used as a sensitive detector.

2. Experimental

2.1 Atomic fluorescence spectrometer

The in-house assembled research grade non-dispersive AFS was employed. Miniature diffusion flame under optimum conditions was used as the atomizer [6].

2.2 Standards and reagents

A stock solution of 1000 mg dm^{-3} was prepared for each arsenic species in deionized water (Ultrapur, Watrex, USA) using following compounds: iAs^{III} from As_2O_3 (Lachema, Czech Republic); iAs^{V} from As stock standard solution (Merck); MMA^{V} from $Na_2CH_3AsO_3.6H_2O$ (Chem. Service, USA); DMA^{V} from $(CH_3)_2As(O)OH$ (Strem Chemicals, USA). A reducing solution of $NaBH_4$ (Fluka) in 0.1% KOH (Lach-ner, Czech Republic) was prepared fresh daily. HCl (Merck) was used for HG. A tris(hydroxymethyl)aminomethane (TRIS) buffer was prepared by mixing of Trizma® hydrochlorid (Sigma) and TRIS (Fluka) to desired concentration and pH (pH was adjusted by KOH (Lach-ner, Czech Republic) and HCl). Mobile phase for ion pair chromatography was 4.7 mmol dm^{-3} tetrabutylammonium hydroxide (Fluka), 2 mmol dm^{-3} malonic acid (Sigma-Aldrich) in 4% methanol (Sigma-Aldrich), pH was adjusted to 5.85 [7]. Mobile phase for anion exchange chromatography was 20 mmol dm^{-3} phosphate buffer (from KH_2PO_4 (Merck) and K_2HPO_4 (Xenon Lodz, Poland)), pH adjusted to 6.00 [8].

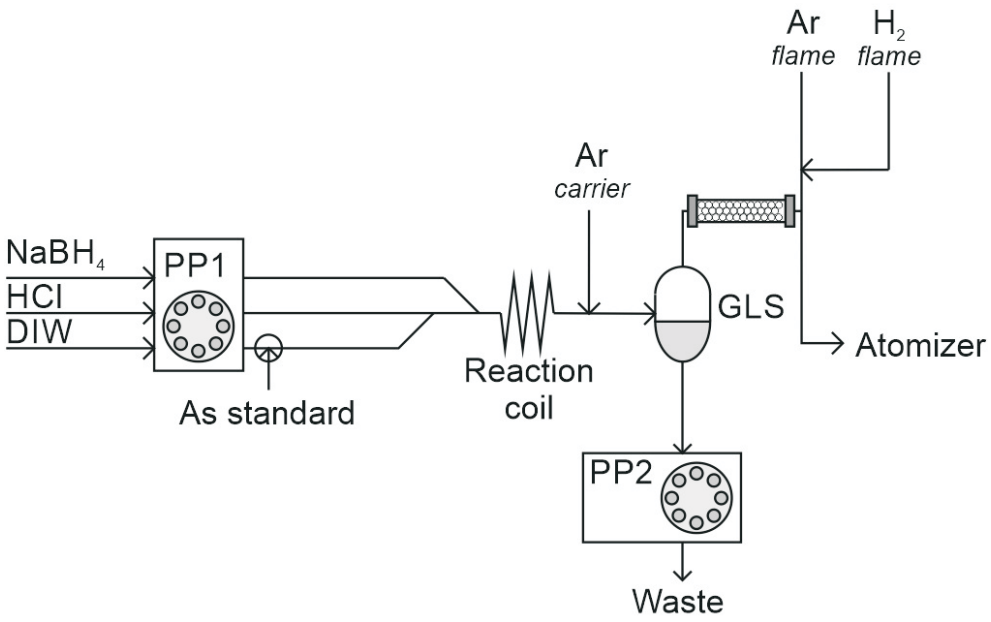


Fig. 1. Flow injection generator: PP1/PP2 – peristaltic pumps, GLS – gas-liquid separator, DIW – deionized water.

2.3 Hydride generators

2.3.1 Flow injection and postcolumn generator

See Fig. 1 for the scheme. $NaBH_4$, HCl and DIW were pumped by a peristaltic pump (Ismatec, Switzerland) at the flow rates of 1 ml min^{-1}. The manifold was built from PTFE tubing and PEEK T-junctions (Cole Parmer, USA). Sample was injected into the flow of DIW by a six-port injection valve (Rheodyne, USA) with 0.6 ml sample loop volume. A reaction coil of different length and i.d. was used. A 5 cm long PEEK capillary of 0.25 mm i.d. (not shown) was employed downstream the reaction coil to introduce 80 ml min^{-1} of Ar. The gaseous phase containing arsanes was separated from the liquid phase in a glass gas-liquid separator (GLS). The second pump was used for waste removal from the GLS. The output from the GLS was connected to the T-junction serving to introduce hydrogen (flame hydrogen) and argon (flame argon) for the atomizer.

HPLC pump (Agilent 1200, USA) with ODS-3 column (15 cm × 4.6 mm, 3 μm particle size) and PRP-X100 column (25 cm × 4.6 mm, 10 μm particle size) were used. The injection valve was substituted with the HPLC outlet (injected volume was 50 μl).

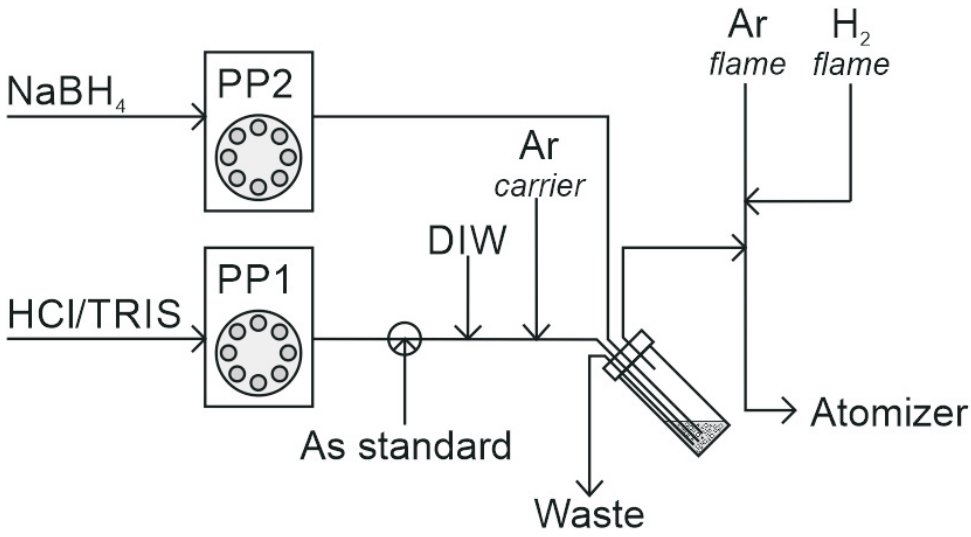


Fig. 2. Batch generator: PP1/PP2 – peristaltic pumps, GLS – gas-liquid separator, DIW – deionized water.

2.3.2 Batch generator (and a cryotrapp)

See Fig. 2 for the scheme. HCl or TRIS buffer were pumped by the peristaltic pump. HCl/TRIS channel lead through the six-port injection valve with 0.6 ml sample loop volume. One T-junction was used to connect DIW channel and another T-junction to connect argon carrier. The HCl/TRIS channel was introduced at the bottom of the GLS (glass vial, total volume of 7 ml). Total volume of 1.2 ml was pumped (0.6 ml sample and 0.6 ml HCl/TRIS) to the GLS. Another peristaltic was used to introduce a solution of 1% $NaBH_4$ also at the bottom of the GLS at flow rate 1 ml min^{-1}. Separate channel was used to remove liquid waste from the GLS after the reaction had been completed. The output from the GLS was connected to the CT (see ref. [5] for details) or directly to the T-junction serving to introduce hydrogen and argon for the atomizer.

3. Results and discussion

3.1 Flow injection generator

The influence of HCl concentration on the sensitivity was tested in the range between 0.25 and 4 mol dm^{-3}. The inorganic arsenic (iAs^{III+V}) sensitivities increased with higher concentration of HCl, but methylated arsenic species (MMA^{V}, DMA^{V}) had maximum sensitivity at lower concentration of HCl and it

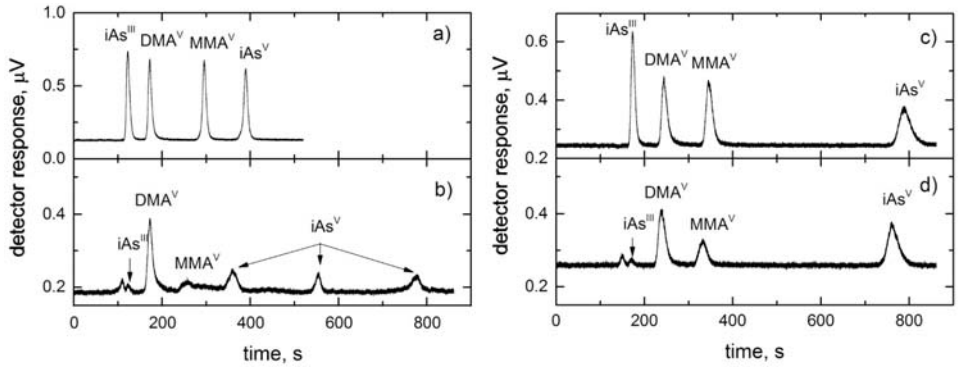


Fig. 3. HPLC–HG–AFS chromatograms using a) ion pair chromatography of aqueous standards (5 ng ml^{-1}), b) ion pair chromatography of a urine sample, c) anion exchange chromatography of aqueous standards (5 ng ml^{-1}) and d) anion exchange chromatography of a urine sample.

decreased at higher concentration of HCl. Concentration of HCl of 2 mol dm^{-3} was chosen as optimum.

Volumes of 0.8, 4.1 and 10.4 ml were tested. The volume of the reaction coil of 4.1 ml was found to be sufficient to increase sensitivity of MMA^V to the maximum. However, iAs^V and DMA^V were not quantitatively converted to their corresponding arsanes. This resulted in their lower relative sensitivity (related to the sensitivity of iAs^{III}, which is assumed to be generated quantitatively, i.e. with 100% generation efficiency). Only 91±1% and 84±1% relative sensitivities were measured for iAs^V and DMA^V, respectively, even for 10.4 ml volume of the reaction coil.

With the reaction coil of 10.4 ml the influence of concentration of $NaBH_4$ was tested in the range between 1 and 3% (m/v). Concentration of 2.5% was found optimal resulting in equal sensitivities for all four arsenic species.

HPLC was connected to HG to separate arsenic species before HG (see Fig. 1). When ODS-3 column was used good separation of arsenic species in DIW solution was found (Fig. 3a), but separation of As species in undiluted urine (NIST 2669) lead to a pronounced tailing of peaks and even multiple peaks for iAs^V were detected (Fig. 3b). 5 times or 10 times dilution of the sample with the mobile phase was necessary to obtain good separation of peaks.

The anion exchange chromatography was tested instead of ion pair chromatography. Very good separation was found for all tested arsenic standards in DIW solution and even in the urine samples (NIST 2669); see Fig. 3c and 3d. Since the same sensitivities for all species were obtained there was the possibility to use a calibration for DMA^V for quantification of the other species. This approach was successfully tested on NIST 2669 (Table 1).

Table 1
Concentration of As species found in NIST 2669 by HPLC–HG–AFS, single species calibration (DMA^V) was used.

Species	Determined ng ml^{-1}	Certified ng ml^{-1}
MMA^V	1.85±0.12	1.87±0.39
DMA^V	3.81±0.22	3.47±0.41
iAs^{III+V}	4.10±0.45	3.88±0.32

3.2 Batch generator

The influence of concentration of HCl on the sensitivity was tested in the range between 0.01 and 6 mol dm^{-3}. The plateau was obtained in the range between 0.5 and 4 mol dm^{-3} where all four arsenic species were generated with the same sensitivity (and probably with 100% generation efficiency). Concentration of 1 mol dm^{-3} was chosen as optimum.

Since it is not possible to generate selectively and quantitatively only iAs^{III} from HCl medium, HG from TRIS buffer was tested. When pH of TRIS buffer was adjusted to 8, only negligible signals (lower than 1.5%) from pentavalent species were measured. Furthermore, sensitivity of iAs^{III} was equal and comparable with the sensitivity obtained when generated from 1 mol dm^{-3} HCl (100±4%).

The effect of addition of $NaBH_4$ (1% solution) on sensitivities of iAs^{III}, iAs^V, MMA^V and DMA^V was tested in the range between 0.08 ml and 2 ml (in the case of HCl medium) and in the range between 0.25 ml and 2 ml (in the case of TRIS buffer medium at pH 8.00). Addition of at least 0.5 ml 1% $NaBH_4$ was found sufficient to generate arsanes quantitatively from all four arsenic species. The addition of 0.75 ml was chosen as optimum.

The CT was connected to the hydride generator to separate the mixture of generated arsanes before the detection by AFS. Ar carrier was changed to hydrogen or helium. Preliminary results with a CT confirm that the peaks corresponding to individual arsanes can be easily separated.

4. Conclusion

The conditions of HG at which arsanes from all four arsenic species were generated with same sensitivity (and thus generation efficiency) were found. Therefore, a single standard calibration can be used for both methods for quantification of all other species. This advantage results in a shorter time for analysis by HPLC–HG–AFS because calibration is constructed only by means of DMA^V, which elutes in the front part of the chromatogram. The accuracy of this calibration approach was successfully demonstrated by the analysis of the certified reference material of urine. The advantage of equal sensitivity for all species obtained in the batch generator coupled to the CT lies in the fact that determination of As species can be performed without further sample pretreatment (pre-reduction) which brings potential risk of contamination at ultratrace levels. Preliminary results suggest that this approach is very promising.

Acknowledgments

GA CR (grant No. P206/14-23532S), Institute of Analytical Chemistry of the CAS, v. v. i. (project No. RVO: 68081715) and by Charles University in Prague (project SVV260205).

References

[1] Francesconi K.A., Kuehnelt D.: *Analyst* **129** (2004), 373–395.
[2] Dědina, J.: Generation of volatile vompounds for analytical atomic spectroscopy. In: *Encyclopedia of Analytical Chemistry.* Meyers R.A. (edit.). Chichester, Wiley 2010, p. 1–39.
[3] Shraim A., Chiswell B., Olszowy H.: *Talanta* **50** (1999), 1109–1127.
[4] Musil S., Matoušek T., Currier J.M., Stýblo M., Dědina J.: *Anal. Chem.* **86** (2014), 10422–10428.
[5] Musil S., Matoušek T.: *Spectrochim. Acta, Part B* **63** (2008), 681–691.
[6] Marschner K., Musil S., Dědina J.: *Spectrochim. Acta, Part B* **109** (2015), 16–23.
[7] Gong Z., Lu X., Cullen W.R., Le X.C.: *J. Anal. At. Spectrom.* **16** (2001), 1409–1413.
[8] Viñas P., López-García I., Merino-Meroño B., Campillo N., Hernández-Córdoba M.: *Chromatographia* **57** (2003) 611–616

Trypsin-based monolithic immobilized enzymatic reactor for proteomic analysis

KINGA MELLER[a, *], DAMIAN GRZYWIŃSKI[a, b], PAWEŁ POMASTOWSKI[a, b], MICHAŁ SZUMSKI[a, b], BOGUSŁAW BUSZEWSKI[a, b]

[a] *Nicolaus Copernicus University in Toruń, Faculty of Chemistry, Department of Environmental Chemistry and Bioanalytics, Gagarina 7, 87-100 Toruń, Poland* ✉ *kingameller91@wp.pl*
[b] *Centre for Modern Interdisciplinary Technologies, Wileńska 4, 87-100 Toruń, Poland*

Keywords
enzymatic microreactors
monolithic support
proteomics

Abstract
The aim of this work was preparation and evaluation of a miniaturized reactor with immobilized trypsin for digestion of proteins. Initially, the monolithic support (copolymer of glycidyl methacrylate and ethylene glycol dimethacrylate) was synthesized in the fused silica capillary. Epoxy group was then modified which resulted in creation of the spacer arm to which trypsin was bound. The first step was aminolysis of the epoxy groups with 1,6-diaminohexane, followed by the attachment of glutaraldehyde to which the enzyme was bound via its amino groups (lysine residues). Finally, the imine bonds were reduced using sodium cyanoborohydride. Monolithic enzyme reactor was tested for its activity using BAEE (N_{α}-benzoyl-L-arginine ethyl ester) and macromolecular protein as substrates. In the case of BAEE the reaction products were separated using nanoHPLC and the efficiency of the reaction was determinated by peak area of the substrate. The hydrolysis products of human lactoferrin were analyzed with MALDI-TOF.

1. Introduction

Proteomics is the field of research dealing with the study of structures and functions of all proteins expressed by a human genome as well as interactions between them. It also includes the identification and quantification of polypeptides, determination of their sequence, spatial structure and post-translational modifications. The problems that can be encountered in proteomic analysis are mainly associated with a complex matrix and small amounts of target proteins in the sample. So, there is a need of using effective, efficient and sensitive analytical methods [1].

Currently, the qualitative analysis of biopolymers is dominated by mass spectrometry and Peptide Mass Fingerprint (PMF) method. It involves the digestion of either all proteins (present in the sample) or previously isolated protein fraction by a specific enzyme (typically trypsin) followed by mass spectrometric determination of the resulting polypeptides which are often characteristic for each individual protein present in the sample. In such a case the identification is

based on a comparison of the obtained MS spectra with the proteomic database. The standard proteolysis procedure includes the enzymatic digestion of the protein in homogeneous solution with dissolved trypsin and in the presence of benzamidine (competitive inhibitor). This method has some limitations, the most important of which are: it is time-consuming (long incubation period of the analyte with the small amount of soluble enzyme which prevents from undesirable autodigestion), a lot of manual work (associated with the increased the risk of contamination and loss of the reaction products), the sample contamination with the fragments from autolysis of the proteases, significant loss of the enzyme as a result of the autodigestion and the difficulty to recover it. One of the solutions used in this area is employing microfluidic reactors with immobilized enzymes (Immobilized Enzyme Reactor, IMER). Microfluidic reactors offer the possibility of rapid digestion of small amount of the analyte, which makes them particularly attractive tools for proteomics or pharmaceutical studies. The IMERs make it possible to carry out highly specific reactions with minimal manual handling, reagent consumption and in a short time. The main important advantage of using IMER is possibility of direct connection to the separation system and mass spectrometer [2–4].

Monolithic organic copolymers are frequently used supports for immobilization of enzymes. This is due to the special properties of the monoliths which include the low back-pressure (which allows the use of the high flow rate) and relatively high surface area (which guarantees the high density of functional groups on the surface of the support). Immobilization of enzymes in microfluidic devices is dominated by covalent attachment which prevents the enzyme from leaching during the flow of the substrate solution through the capillary IMER. This approach allows to obtain the highly durable and biologically inert system. Additionally, the immobilization of the enzymes on the solid support leads to increase their stability and resistance to denaturation [4–5].

2. Experimental

2.1 Reagents and chemicals

Fused-silica capillaries (150 μm i.d. × 375 μm o.d.) were obtained from Polymicro Technologies; 3-(trimethoxysilyl)propyl methacrylate (γ-MAPS), glycidyl methacrylate (GMA), ethylene dimethacrylate (EDMA), cyclohexanol, 1-dodecanol, 1,6-hexanediamine, glutaraldehyde, sodium cyanoborohydride, N_{α}-Benzoyl-L--arginine ethyl ester (BAEE), benzamidine were obtained from Sigma-Adrich. Trypsin was obtained from Biological Industries (trypsin: 365.47 USP-u/mg, chymotrypsin: 151.23 USP-u/mg); azobisisobutyronitrile (AIBN) was from Fluka.

2.2 Instrumentation

A syringe pump type NE-1002X (New Era Pump Systems) was used to push the reagents (during the modification) and substrate solution through the μ-IMER. In the case of carrying out the reaction at a constant temperature the thermostat (Julabo, type F25) connected to a specially designed heat exchanger was used. All chromatographic experiments were performed in a nanoHPLC chromatograph system consisting of a 1100 Series Agilent Technologies pump, a 10-port nanoLC valve (model C72MX-6690D Vici-Valco) and a Crystal 100 UV-Vis detector (Thermo Separation Products). The system was controlled with the Clarity software (DataApex, Prague Czech Republic).

2.3 Capillary pretreatment

Initially, the capillary (150 μm i.d. × 375 μm o.d.) was rinsed with acetone and dichloromethane and then dried with an inert gas (nitrogen). The inner wall of the fused silica capillary was treated with 1 M NaOH solution at 100 °C for 3 h (etching). Surface modification (silanization) was carried out with 10% γ-MAPS solution in toluene. The reaction was performed at room temperature for 2 h.

2.4 The preparation of the monolithic support

The polymerization mixture consisted of 24% GMA, 16% EDMA (w/w), 1% AIBN (w/w with respect to the total monomer content) and porogen solution containing 70% cyclohexanol and 30% 1-dodecanol (60% with respect to the total mass of the mixture). The prepared polymerization mixture was sonicated, shaked and purged with nitrogen. The polymerization was carried out in a water bath with stirrer at 80 °C for 24 h.

2.5 Immobilization of trypsin

The aminolysis of epoxy rings was carried out with 10% 1,6-hexanediamine solution in 0.1 M sodium bicarbonate buffer for 2.5 h at 80 °C. The glutaraldehyde attachment was performed using 10% glutaraldehyde solution in 0.05 M PBS for 3 h at room temperature. The immobilization was carried out with 3.5 mg/ml trypsin solution in PBS with benzamidine addition for 24 h at 4 °C. Finally, the imine bonds were reduced with $NaCNBH_3$ for 2 h at room temperature (Fig. 1).

2.6 μ-IMER evaluation

The yield of the reaction carried out in μ-IMER was determined by hydrolysis of the standard substrate for trypsin N_α-benzoyl-L-arginine ethyl ester (BAEE). The substrate solution (in 0.05 mM phosphate-buffered saline (PBS), pH = 8) was

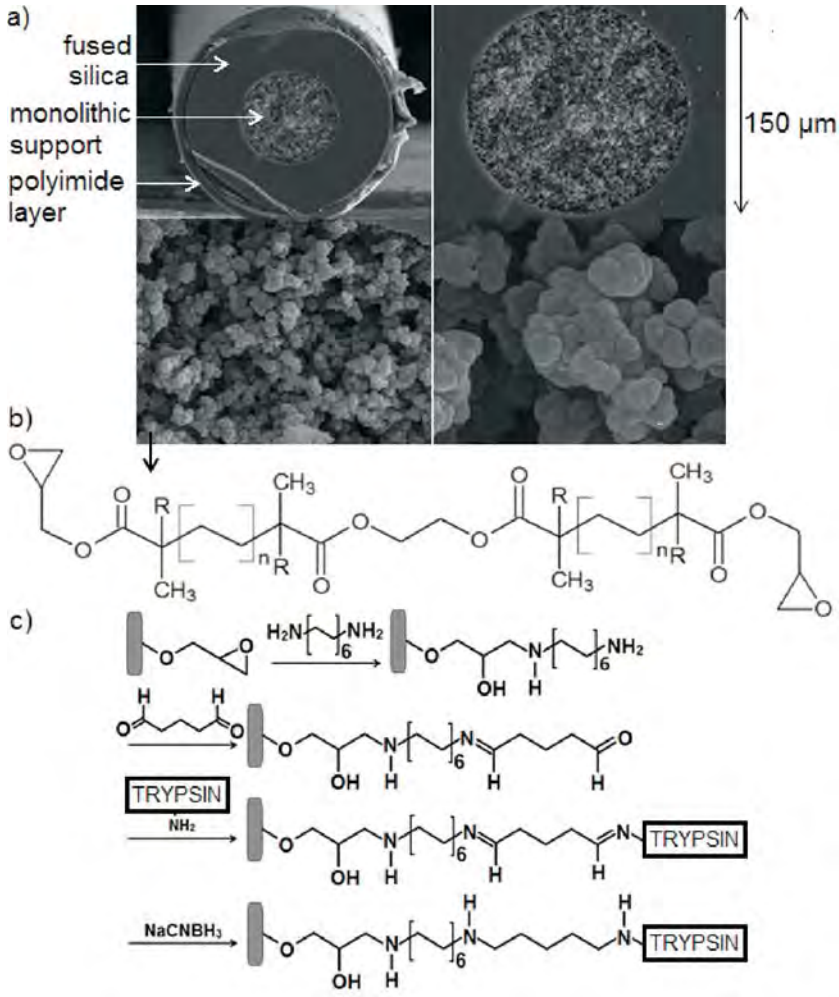


Fig. 1. a) Scanning electron micrograph of monolithic GMA-co-EDMA, b) the chemical formula of copolymer c) scheme immobilization of trypsin on the support.

passed through a µ-IMER using the syringe pump and the eluate from the µ-IMER continuously passing through the injection loop was sequentially introduced into the chromatographic column. The reaction product, N_{α}-benzoyl-L-arginine (BA), was separated from the BAEE and the degree of hydrolysis was determined based on the peak area of the substrate using the calibration curve. The separation was performed using a nanoHPLC column packed with octadecyl stationary phase. The mobile phase consisted of water (with 0.1% trifluoroacetic acid (TFA)) and acetonitrile (ACN with 0.1% TFA) at a flow rate of 2.0 µl/min. The chromatographic process was started from 20% of ACN content in a mobile phase which increased to final 40% ACN. The detection was performed at the wavelength of $\lambda = 223$ nm.

2.7 Protein digestion and MALDI-TOF identification

1.8 mg of lactoferrin was dissolved in 500 µl of a buffer (50 mM ammonium bicarbonate buffer, pH = 8.05). The protein solution was passed through a µ-IMER using a syringe pump at 37 °C and at a flow rate of 0.75 µl/min (residence time equalled 68 s). The eluate from the µ-IMER was collected in a plastic vial and then analysed using a mass spectrometer MALDI-TOF. PMF spectra of lactoferrin was recorded in the positive mode using an acceleration voltage of 25 kV. The matrix used during the sample preparation was the acid α-cyano-4-hydroxycinnamic acid (CHCA).

3. Results and discussion

The degree of hydrolysis of the ester (BAEE) was calculated on the basis of the substrate concentrations in the solutions before ($c_{\mathrm{BAEE,\ wz}}$ [mM]) and after (c_{BAEE} [mM]) reaction carried out in the µ-IMER (determined by the calibration curve) [mM] by the following formula:

$$\%\mathrm{H} = \frac{c_{\mathrm{BAEE,wz}} - c_{\mathrm{BAEE}}}{c_{\mathrm{BAEE,wz}}}[\%] \qquad (1)$$

The BAEE solutions in the range of concentration 0.5–75,0 mM were passed through the µ-IMER at the different flow rates: 0.25, 0.50, 0.75 and 1.00 [µl/min] (Fig. 2). Additionally, the 25.0 mM BAEE solution was passed through the µ-IMER at the different temperatures: 15, 20, 30 and 37 °C (Fig. 3).

The results indicate that the degree of hydrolysis BAEE decreased with the increasing of both flow rate and substrate concentration. It was due to the reduced contact time between the enzyme and substrate, determining the possibility of

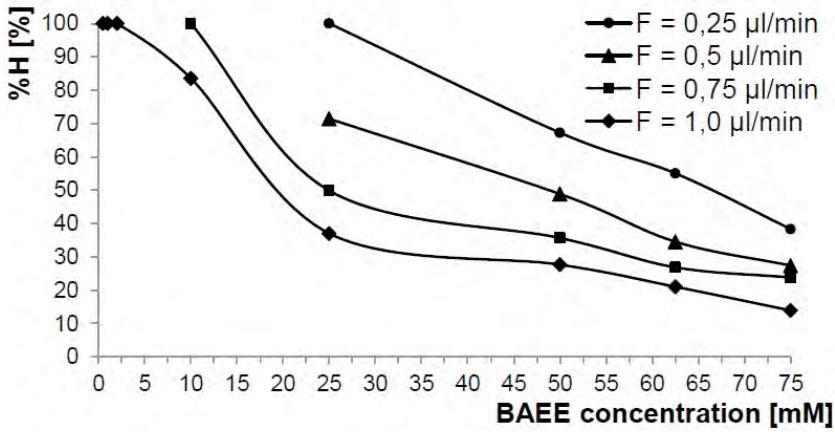


Fig. 2. The effect of BAEE concentration on degree of hydrolysis at different flow rates of the substrate solution.

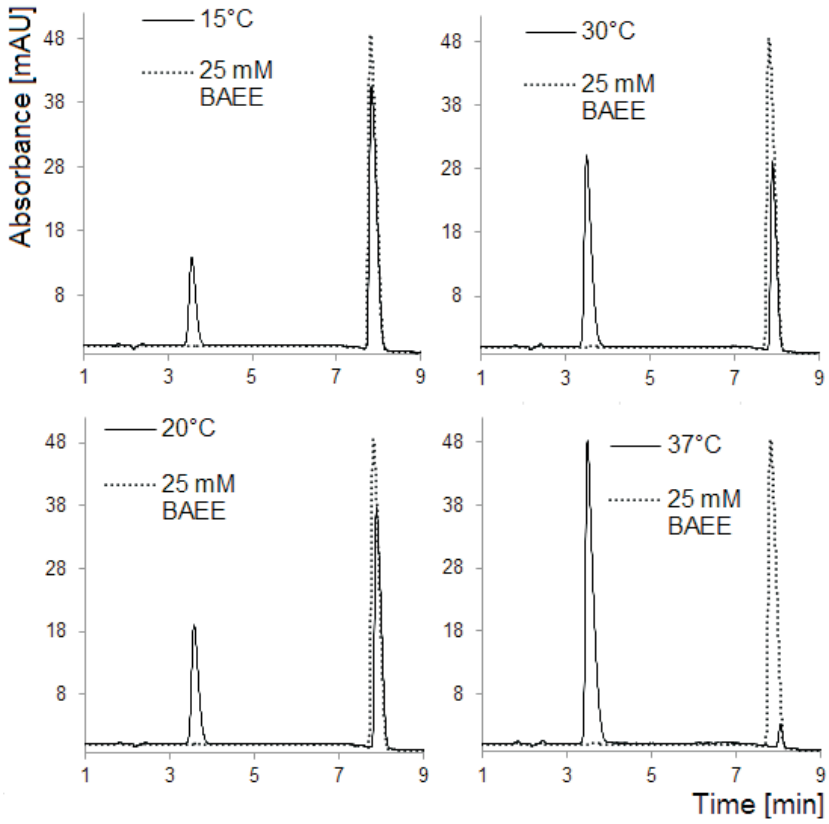


Fig. 3. The comparison of chromatograms obtained by the direct introduction of 25 mM substrate solution onto a separation column, and after passing it through the μ-IMER at F = 1 μl/min at different temperatures.

supramolecular fitting of the active sites of the biocatalyst to the place of its activities. It was also observed that the degree of hydrolysis of BAEE increased with the temperature and reached the highest value at 37 °C.

The results obtained for lactoferrin digest (analysed by MALDI-TOF) indicate that the protein was completely hydrolysed (no signal from intact lactoferrin on the MS spectra; Fig. 4). However, in the spectrum recorded only three characteristic signals (resulting from trypsin activity) and a large number of other signals at low values of *m/z* (derived from the polypeptides of different molecular weight). Probably, the reason of the observed phenomenon is the presence (in the purchased trypsin) of significant amounts of chymotrypsin. Nevertheless, the results confirm the immobilization of trypsin on a support and the maintaining of its specific activity (occurrence of characteristic signals on the MS spectrum; Fig. 5). The non-specific operation of μ-IMER was entirely due to the activity of chymotrypsin.

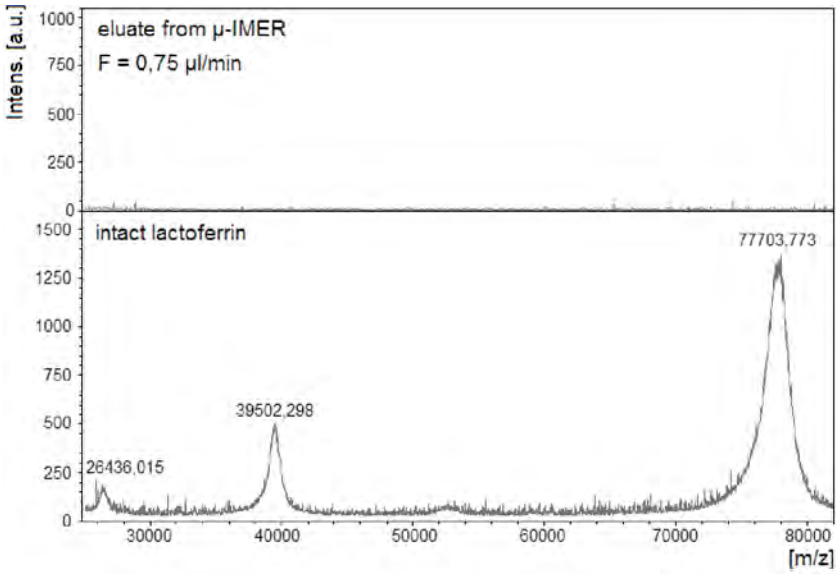


Fig. 4. Comparison of mass spectra of the eluate from the μ-IMER and the native lactoferrin solution.

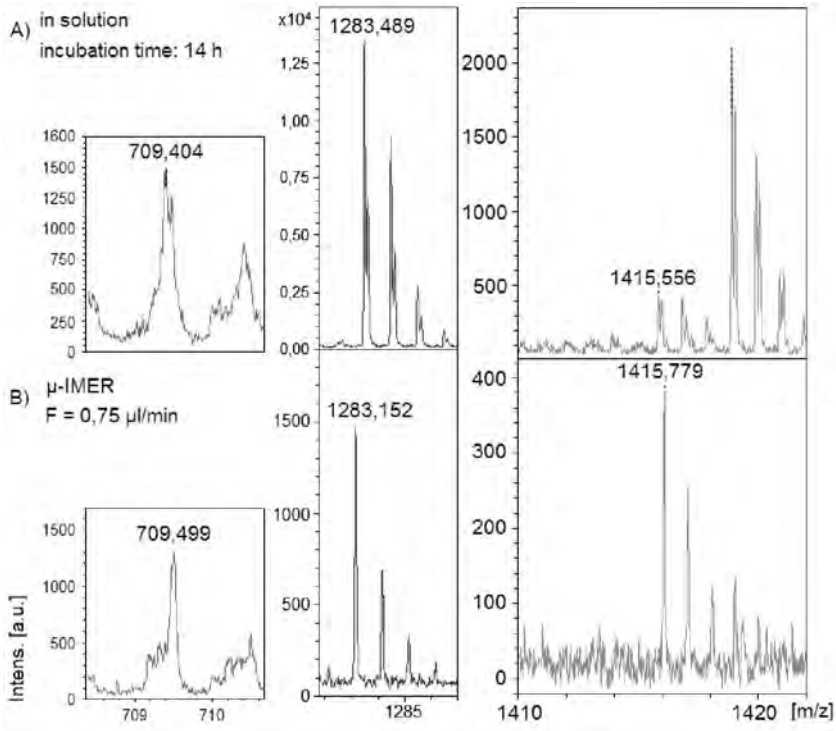


Fig. 5. Comparison of the mass spectra fragments containing the characteristic signals:
(A) lactoferrin digested with trypsin in a solution (incubation time: 14 h)
(B) lactoferrin digested with μ-IMER (residence time: 68 s).

4. Conclusions

The prepared μ-IMER showed a high enzymatic activity with regard to both low and high molecular weight substrates (BAEE and lactoferrin). It allows to carry out the reaction at the short time without significant loss of efficiency

Acknowledgments

The work was financed by the National Science Center (Krakow, Poland) grant no. 2013/11/N/-ST4/01837. The financial supports of the European Regional Development Fund under the ROP for the years 2007-2013: project no. RPKP.05.04.00-04-003/13 and Kujawsko-Pomorskie Voivodship Budget "Krok w przyszłosc" are also kindly acknowledged.

References

[1] Ma J., Zhang L., Liang Z., Shan Y., Zhang Y.: *Trends Anal. Chem.* **30** (2011), 691–702.
[2] Sproß J., Sinz A.: *Anal. Bioanal. Chem.* **395** (2009), 1583–1588.
[3] Yao C., Qi L. Hu W., Wang F., Yang G.: *Anal. Chim. Acta* **692** (2011), 131–137
[4] Duan J., Liang Z., Yang C., Zhang J., Zhang L., Zhang W., Zhan Y.: *Proteomics* **6** (2006), 412–419.
[5] Ma J., Zhang L., Liang Z., Zhang W., Zhang Y.: *J. Sep. Sci.* **30** (2007), 3050–3059.

HPLC enantioseparation on derivatized cyclofructan chiral stationary phases

MARIANNA MOSKAĽOVÁ*, TAŤÁNA GONDOVÁ

P. J. Šafárik University, Faculty of Science, Department of Analytical Chemistry, Moyzesova 11, 040 01 Košice, Slovak Republic ✉ *marianna.moskalova@gmail.com*

Keywords
chiral separation
cyclofructan
enantioseparation
HPLC

Abstract
The direct HPLC enantiomeric separation of new racemic compounds was studied on a cyclofructan-based chiral stationary phases under normal phase mode. The various experimental variables were investigated for the optimization of the separation.

1. Introduction

High-performance liquid chromatography is commonly used as an effective analytical method for the separation of enantiomers. Recently, derivatized cyclofructans, with aliphatic and aromatic functionalities have been developed as bonded chiral stationary phases for HPLC. Derivatized cyclofructan-based chiral stationary phases provide excellent and unique selectivities for a variety of racemic mixtures. These columns are multimodal, i. e. they can be used in normal phase, polar organic, and reversed-phase modes. They also show higher stability by using common organic solvents and more aggressive acidic and basic additives [1].

Phytoalexins are secondary metabolites produced by plants. These natural compounds and their derivatives have received attention because of their biological activities, including antibacterial, chemopreventive and significant antiproliferative properties [2].

The present study deals with the comparison of direct LC enantiomeric separation of new racemic analogs of phytoalexins on two derivatized-cyclofructan based chiral stationary phases, Larihc CF6-RN and Larihc CF7-DMP, under normal phase mode.

2. Experimental

2.1 Reagents and chemicals

New racemic derivatives of indole phytoalexins were synthesized at the Department of Organic Chemistry (P. J. Šafárik University, Košice). HPLC grade organic solvents were purchased from Merck.

2.2 Instrumentation

HPLC experiments were performed on Infinity 1200 HPLC system consisted of a pump, an injection valve Rheodyne with a 20 µL sample loop and an LC UV-visible detector with variable wavelength. Chiral separations were performed on Larihc CF6-RN and Larihc CF7-DMP columns (250 × 4.6 mm, 5 µm; University of Texas, Arlington, USA).

3. Results and discussion

Direct HPLC separation was performed on two derivatized cyclofructan chiral stationary phases in the normal phase mode. The mobile phases consisted of *n*-hexane and polar modifier in various ratios. The effects of the type and concentration of the polar modifier as well as the nature and concentration of mobile phase additives (acidic and basic) on the chromatographic parameters were tested. The results obtained on two different chiral stationary phases were compared (Fig. 1.). The influence of column temperature on enantioselectivity was also examined.

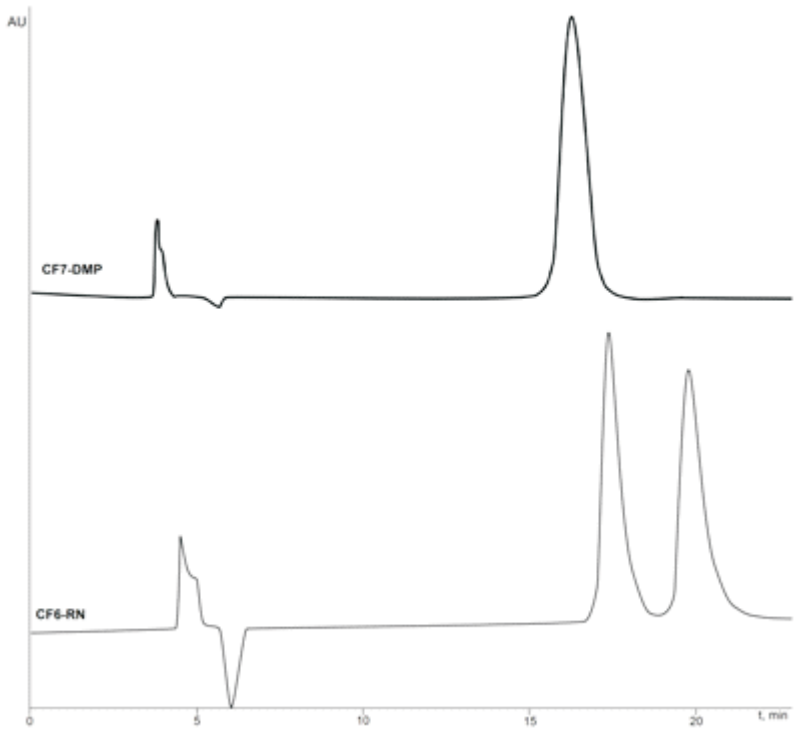


Fig. 1. Separation of chiral compound on two different chiral stationary phases (mobile phase: *n*-hexane:2-propanol (90:10 v/v), temperature 20 °C, detection 250 nm).

4. Conclusions

Two derivatized cyclofructan chiral stationary phases were used for the enantiomeric separation of new bioactive racemic compounds under normal phase conditions. The results obtained were evaluated and compared. It was shown, that the chiral separation was affected by the type of chiral selector, mobile phase composition as well as the structure of tested analytes.

Acknowledgments

This work was supported by the internal grant VVGS-PF-2014-448.

References

[1] Sun P., Wang Ch., Breitbach Z.S., Zhang Y., Armstrong D.W.: *Anal. Chem.* **81** (2009), 10215–10226.

[2] Kutschy P., Salayová A., Čurillová Z., Kožár T., Mezencev R., Mojžiš J., Pilátová M., Balentová E., Pazdera P., Sabol M., Zburová M.: *Bioorg. Med. Chem.* **17** (2009), 3698–3712.

The use of electronic nose for sunflower and rapeseed oil classification

Natalia Nosal*, Tomasz Dymerski, Jacek Namieśnik

Department of Analytical Chemistry, Faculty of Chemistry, Gdańsk University of Technology, Narutowicza 11/12 Street, 80-223 Gdańsk, Poland ✉ *natalia.nosal@pg.gda.pl*

Keywords
edible oils
electronic nose
Principal Component Analysis

Abstract
The electronic nose is an analytical device often used in food industries to examine the authenticity of their products. The use of the electronic nose allows for a rapid assessment of the quality of food. The oil samples from sunflower and rapeseed were used in this study. Both oil samples were kept inside the thermostat incubator at different ranging from 20 °C to 180 °C with increment of 40 °C. Principal Component Analysis were employed to evaluate the data. The results confirmed that the electronic nose was successfully used to distinguish the oil samples from sunflower and rapeseed.

1. Introduction

Edible oil is a mixture of chemical compounds, such as essential fatty acids (saturated and unsaturated), their derivatives and terpenes, sterols, and carotenoids that are soluble in organic solvents i.e. hexane, methanol, ethyl ether and chloroform [1–5]. A number of research proved that edible oil provides some essential compounds for the diet and contributes positively to human health [6–13]. However, edible oil is at the risk of adulteration and deterioration that generates some disadvantages for the consumer [14]. Therefore, the authenticity of edible oils on the market should be controlled as well as their quality on the production level. Given this fact, it constitutes a threat to human health, in particular those with food allergies (some components of edible oils have sensitizing properties).

The electronic nose is widely applied in food industries: spirits, wine, fruit, vegetable, and dairy food production. This devise can be used to detect food adulteration and evaluate the freshness of food products thus useful for food quality control [15–21]. Hence, an analytical tool employing electronic nose is necessary to evaluate the quality profile of edible oil products. In order to achieve this purpose, the electronic nose based on ultra-fast gas chromatography was used to evaluate and distinguish the volatile fraction profiles of oil samples originated from sunflower and rapeseed.

2. Experimental

2.1 Samples

The edible oils used in this study were obtained from Polish market including refined Wyborny rapeseed oil (Jeronimo Martines S.A, Poland) and Słonecznikowy sunflower oil (Tesco SP. Z. O. O, Poland). The samples were kept in plastic packaging with light protection at room temperature.

2.2 Analytical procedure

The samples were placed into 20 cm^3 vial and closed with cap made from a teflon--silicone membrane. The samples were then heated for 24 hours at different temperature ranging from 20 °C to 180 °C with an interval of 40 °C. The analyses were in triplicate.

2.3 Instrumentation

The prototype of heating device with temperature control was used in this study developed at the Department of Analytical Chemistry, Faculty of Chemistry, Gdańsk University of Technology. The analyses were carried out using Heracles II electronic nose equipped with HS 100 auto-sampler (Alpha M.O.S., Toulouse, France). In Table 1. the list of experimental conditions of electronic nose device was presented.

Table 1
Working parameters of the electronic nose used for sunflower and rapeseed oil classification.

Autosampler	
Incubation time	900 s
Agitation speed	500 rpm
Analyzer	
Injection volume	2500 µl
Injection speed	250 µl/s
Trap	
Trapping temperature	40 °C
Split valve	1 ml/min
Chromatographic temperature program	
40 °C (4 s) $\xrightarrow{3\ °C/s}$ 270 °C (30 s)	

2.4 Data analysis and chemometrics

The data obtained from the analysis were calculated using a chemometric method, namely Principal Component Analysis (PCA), via a data analysis software Alpha-Soft version 12.4.

3. Results and discussion

The PCA has been used to evaluate the data obtained from the analysis of oil samples after exposure to different temperatures (20 °C, 60 °C, 100 °C, 140 °C, and 180 °C). The results from PCA analysis is shown in Fig. 1. Regarding to the obtained results it can be noticed that independently on the temperature in which the incubation process was provided the discrimination of both types of oils can be achieved using electronic nose device. The reduction of input data was not

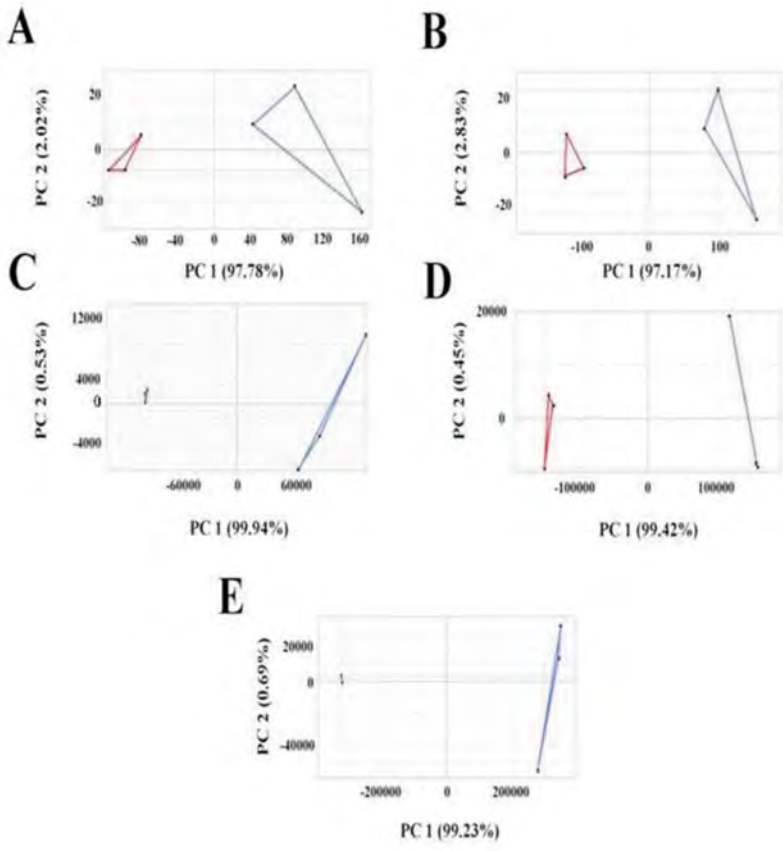


Fig. 1. The PCA results obtained for sunflower (marked in red colour) and rapeseed (marked in blue color) oil samples incubated in temperature: (A) 20 °C, (B) 60 °C, (C) 100 °C, (D) 140 °C, (E) 180 °C.

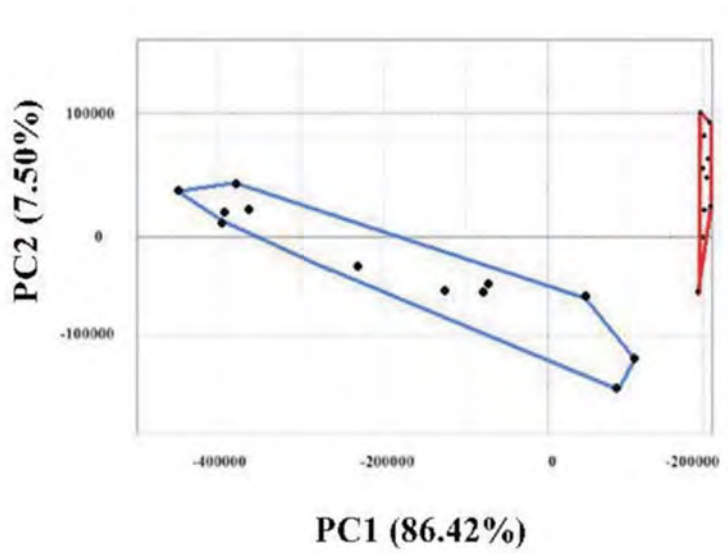


Fig. 2. The PCA result obtained for sunflower (marked in red colour) and rapeseed (marked in blue color) oil samples incubated in the range of temperature from 20 °C to 180 °C with interval of 40 °C.

significant. For each PCA result output data consisted more than 97% of input data. It proves a proper PCA loadings were applied. The precision of the results is higher for sunflower samples. The incubation temperature has great influence on differentiation of these two types of samples. Moreover, it can be explained in higher temperatures degradation process is in much more advanced stage. Then the differences in the composition of these two types of samples are more distinct. In higher temperatures low molecular weight compounds, i.e. aldehydes, ketones, derivatives of carboxylic acids, and many more substances are created as an artefacts. On the other hand need to be noticed that regarding to the Fig. 1 all samples during one experiment was done by the use of a set of samples incubated in one fixed temperature and it was a basis to provide one single PCA result. In this case the samples incubated in different temperatures were not compared. For this reason another single PCA result was provided for the samples incubated in whole above mentioned range of temperature (from 20 °C to 180 °C with intervals of 40 °C). This PCA score is presented in Fig. 2. Regarding to the Fig. 2 it can be observed that the reduction of data was increased. In this case more than 86% of inlet data constitute the outlet data. Despite of that fact full discrimination of both types of samples was also achieved independently in which temperature samples were incubated.

4. Conclusions

Regarding to the provided study it is confirmed the electronic nose is a powerful tool which allows to conduct unambiguous classification of sunflower and

rapeseed oils. Using the elaborated analytical procedure and above-mentioned device it is possible to differentiate both types of samples incubated in room temperature (20 °C), which is nearly the same temperature when customer is purchasing the oils from supplier. Moreover, the authenticity of oil can be also confirmed when the incubation temperature is not known. This can be helpful for measurement in which fried oil is used. The range of 20 °C to 180 °C was used in this research due to the conditions used during the frying on oil in home, restaurants and other catering outlets.

Summarizing, provided work gives a great opportunity to increase the state of the knowledge on food safety in terms of the topic of allergy and it can be a background for continuation of works connected with electronic nose application in terms of thermal degradation of oils.

References

[1] O'Keeffe S. F.: Nomenclature and classification of lipids. In: *Food Lipids. Chemistry, Nutrition, and Biotechnology.* Akoh C. C., Min D. B. (edits). New York, Marcel Dekker 2002, p.19–58.
[2] EN ISO 6886:2008 *of Vegetable and Animal Oils and Fats. Determination oxidation stability (accelerated oxidation).*
[3] Roman O., Heyd B., Broyart B., Castillo R., Maillard M.N.: *LWT-Food Sci. Technol.* **52** (2013), 49–59.
[4] Moros J., Roth M., Garrigues S., de la Guardia M.: *Food Chem.* **114** (2009), 1529–1536.
[5] Wei F., Hua N., Lv X., Donga X.-Y., Chen H.: *J. Chromatogr. A* **1404** (2015), 60–71.
[6] Escuderos M.E., García M., Jiménez A., Horrillo M.C.: *Food Chem.* **136** (2013), 1154–1159.
[7] Dupuy N., Le Dreäau Y., Ollivier D., Artaud J., Pinatel C., Kister J.: *J. Agric. Food Chem.* **53** (2005), 9361–9368.
[8] Ruiz-Samblása C. , Marinib F., Cuadros-Rodrígueza L., González-Casadoa A.: *J. Chromatogr. B* **910** (2012), 71–77.
[9] Zhanga L., Tiana F.C.: *Anal. Chim. Acta* **816** (2014), 8–17.
[10] Osorio M.T., Haughey S.A., Elliott C.T., Koidis A.: *Food Res. Int.* **60** (2014), 66–75.
[11] Ontañon I., Sanz J., Escudero A., de Marcos S., Ferreira V., Galbán J.: *J. Chromatogr. A* **1388** (2015), 52–59.
[12] Peris M., Escuder-Gilabert L.: *Anal. Chim. Acta* **638** (2009), 1–15.
[13] Vaskova H., Buckova M.: *Procedia Eng.* **100** (2015), 630–635.
[14] Nunes C.A.: *Food Res. Int.* **60** (2014), 255–261.
[15] Hai Z., Wang J.: *Sensor Actuat. B* **119** (2006), 449–455.
[16] Berna A.: *Sensors* **10** (2010), 3882–3910.
[17] Scott S.M., James D., Ali Z.: *Microchim. Acta* **156** (2007), 183–207.
[18] Wardencki W., Biernacka P., Chmiel T., Dymerski T.: *Proc. ECOpole.* **3** (2009), 273–279.
[19] Dymerski T., Gębicki J., Wardencki W., Namieśnik J.: *Sensors* **13** (2013), 15954–15967.
[20] Dymerski T., Gębicki J., Wardencki W., Namieśnik J.: *Sensors* **14** (2014), 10709–10724.
[21] Dymerski T., Chmiel T., Wardencki W.: *Rev. Sci. Instrum.* **82** (2011), 1–32.

UV-photochemical volatile compounds generation from selenium species

Eliška Nováková*, Petr Rychlovský

Charles University in Prague, Faculty of Science, Department of Analytical Chemistry
Hlavova 8/2030, 128 43 Prague 2, Czech Republic ✉ *novakoe1@natur.cuni.cz*

Keywords
selenium
volatile compounds generation
UV-photochemical reactor

Abstract
The contribution summarizes the results of TiO_2/UV-photochemical vapour generation of volatile form of Se from various selenium species. This emerging method of vapour generation uses the knowledge achieved in the water treatment processes for a very sensitive determination of Se species. It is particularly efficient in the determination of inorganic selenium; very good limits of detection in the pg·mL^{-1} region were obtained for selenate and selenite with QT-AAS detection. Nevertheless this technique is also suitable for very sensitive determination of organic Se species. The methods accuracy was assessed by the analysis of spiked matrices (groundwater and mineral water samples) and by an interference study. Furthermore, experiments were carried out to find the wavelength and energy responsible for the UV-photo-chemical generation of Se volatile compound and to obtain some information about the reactions taking place during the reduction/generation process.

1. Introduction

Vapour generation techniques are widely applied in elemental determinations to increase the sensitivity of analytical atomic spectrometric methods and suppress the spectral interferences. [1] The emerging UV-photochemical vapour generation offers several advantages compared to the widely accepted chemical vapour generation. In the case of selenium, the major advantage is an on-line pre-reduction of the selenate ion using the TiO_2 catalyst. [2] Another general advantages lie in significantly lower performance cost, simple operation and avoidance of an environmentally harmful sodium tetrahydroborate reagent. Furthermore, negligible evolution of hydrogen compared to other methods of vapour generation improves stability of plasma ionization sources (ICP, DBD, etc.) when they are employed. [3] The use of low molecular organic hole scavengers (usually acids) instead of sodium tetrahydroborate allows to further decrease the limits of detection, which are currently limited by the purity of the reductant. Wide range of elements seem to be accessible by this generation technique: As, Bi, Co, Fe, I, Hg, Ni, Sb, Se, Sn, Pb, Te with the possibility of expanding the scope to cover other transition metals (e.g. Ag, Au, Cu, Pd, Rh) or even non-metals (S) (ref. [4–6]).

Selenium volatile compounds generation is widely studied [7–13] because Se is a useful model analyte for these techniques. Otherwise it is also a toxicologically interesting element causing concern especially in environmental toxicology.

2. Experimental

2.1 Reagents and chemicals

Deionized water used in the preparation of solutions was prepared by the MilliQplus system (Millipore, USA). All chemicals used were of the p.a. purity or better. Sodium selenite pentahydrate, sodium selenate decahydrate, methyl-Se--cysteine hydrochloride, Se-urea and Se-L-methionine (all from Sigma-Aldrich) were used to prepare standard solutions of 100 $\mu g \cdot L^{-1}$ of Se in the given form. Formic, acetic and propionic acids (all Sigma-Aldrich, ACS reagent purity) were used as hole scavengers/generation medium. Nanocrystaline TiO_2 (Sigma-Aldrich) suspended in a matching acid was continuously added to the irradiated reaction mixture. $K_2S_2O_8$ was used to release Se from its organic substances. Argon (4.8, Linde Gas, CR) and H_2 (3.0, Linde gas, CR) were used as the carrier and atomization gases. Diluted standard solutions (for atomic spectrometry) of As, Sb, Cu, Ni, Fe, Na, Ca and Mg (Analytika, CR) as well as solutions of HCl, HNO_3 (Merck), NaCl, $NaNO_3$ (Lach-ner, CR) were used to evaluate the influence of the concomitant ions on the determination of Se.

2.2 Instrumentation

The photochemical reactor used in this study consisted of a PTFE tubing (0.8 mm i.d., 1.58 mm o.d.) wrapped around a low pressure mercury UV lamp (20 W, 253.7 nm). A Solaar 939 atomic absorption spectrometer (Unicam, UK) equipped with an externally resistance-heated (EHT, RMI, Czech Republic) quartz tube atomizer (T-shaped, optical axis arm length 120 mm and internal diameter 8 mm, inlet arm length 100 mm and internal diameter 5 mm) was used in this work. Measurements were carried out at the 196.0 nm line (0.5 nm bandwidth). A MasterFlex L/S peristaltic pump (Cole-Parmer, USA) was used for pumping and withdrawing of all solutions. The apparatus used in this work is shown in Fig.1.

3. Results and discussion

Experimental conditions were first tested and optimized for the determination of the inorganic forms selenite and selenate. The optimized reaction conditions included the type and the concentration of the low molecular weight organic acid, concentration of the titanium dioxide suspension, reactor length, sample volume, flow rate of the organic acid and flow rates and introduction positions of the carrier and atomization gases. Superior limits of detection on the $pg \cdot mL^{-1}$ level

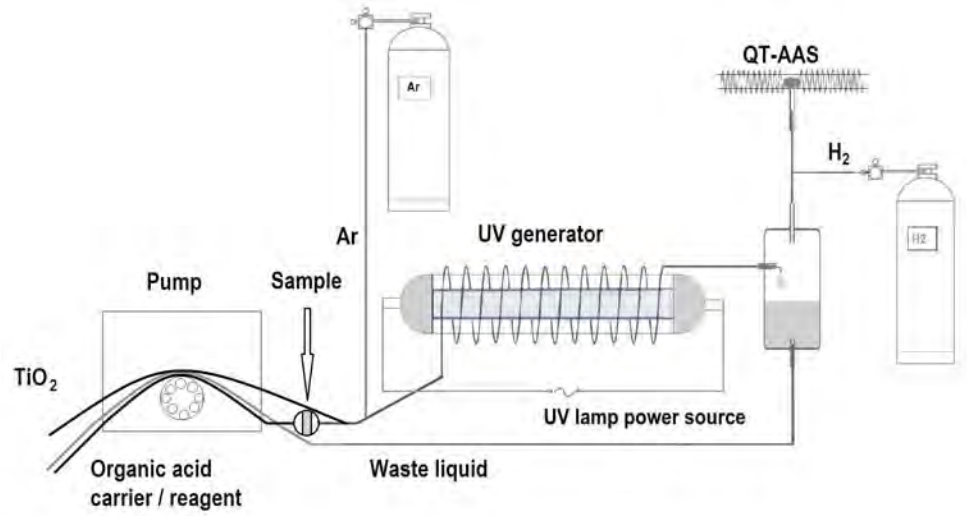


Fig. 1. Apparatus for the flow injection determination of Se by the TiO_2/UV-photochemical vapour generation with QT-AAS detection.

were achieved for 100 µL of selenite and selenate, which are comparable with the results generally attained with MS or AFS detection. [9, 10] On the other hand, UV-photochemical generation from at least some organoSe compounds (methyl--Se-cystein, Se-methionine) is a more complicated matter. It has been found possible when using the TiO_2/UV generation system without further additives; nevertheless the acquired sensitivity is notably worse compared to the inorganic Se compounds due to an incomplete reaction. This was proved by the addition of $K_2S_2O_8$, which led to an increase of generation efficiency and analytical signals of organic Se compounds. Finding a suitable reaction modifier will be necessary for further improvement of this technique.

The methods accuracy was assessed by the analysis of spiked matrices (groundwater and mineral water samples) and by an interference study. The later included other hydride forming elements (As^{III}, Sb^{III}), transition metals (Cu^{II}, Ni^{II}, Fe^{III}), some of the major constituents of water samples (Na^{I}, Ca^{II}, Mg^{II}) and the most commonly occurring acids and their sodium salts (HCl, NaCl, HNO_3, $NaNO_3$). It seems that the UV-photochemical generation of Se is liable to interferences, which can be partly caused by the purity of the chemicals used. However, no carry-over effects were observed. Last but not least, UV lamps with different emission spectra were tested for the UV generation of inorganic Se with and without TiO_2 suggesting that the 254 nm line is responsible for the generation reaction.

4. Conclusions

Sensitive determination of Se in the form of the selenite ion, selenate ion, methyl-Se-cysteine and Se-methionine has been achieved by the UV-photochemical volatile compounds generation. The on-line generation of volatile Se compounds from selenate ion required a reduction step, which was attained by the addition of TiO_2 slurry. This approach led to the achievement of superior limits of detection. However, at the same time the sensitivity of organic Se species determination at the given conditions was notably lower due to an incomplete reaction. Nevertheless, in the end very good limits of detection below ng·mL-1 region were obtained for the selected forms of Se.

The drawback of this approach seems to be the interference of concomitant ions, which would be studied further with the aim of reducing its influence. Experiments done with different UV lamps suggest that the wavelength responsible for the UV-photochemical generation may be the 254 nm line.

Acknowledgments

The project was financially supported by the Grant Agency of the Charles University in Prague (GA UK No. 228214).

References

[1] Dědina J., Tsalev D.L.: *Hydride Generation Atomic Absorption Spectrometry.* New York, Wiley 1995.
[2] Kikuchi E., Sakamoto H.: *J. Electrochem. Soc.* **147** (2000), 4589–4593.
[3] Cai Y., Li S., Dou S., Yu Y., Wang J.: *Anal. Chem.* **87** (2015), 1366–1372.
[4] Zheng, C., Sturgeon R.E., Brophy C., Hou. X.: *Anal. Chem.* **82** (2010), 3086–3093.
[5] Wu P., He L., Zheng C., Hou X., Sturgeon R.E.: *J. Anal. At. Spectrom.* **25** (2010), 1217–1246.
[6] Guo X., Sturgeon R.E., Mester Z., Gardner G.J.: *Anal. Chem.* **76** (2004), 2401–2405.
[7] Guo X., Sturgeon R.E., Mester Z., Gardner G.J.: *Anal. Chem.* **75** (2003), 2092–2099.
[8] Sun Y., Chang Y., Su C.: *Anal. Chem.* **78** (2006), 2640–2645.
[9] Zheng C., Wu L., Ma Q., Lv Y., Hou X.: *J. Anal. At. Spectrom.* **23** (2008), 514–520.
[10] Li H., luo Y., Li Z., Yang L., Wang Q.: *Anal. Chem.* **84** (2012), 2974–2981.
[11] Deng D., Zhou J., Ai X., Yang L., Hou X., Zheng C.: *J. Anal. At. Spectrom.* **27** (2012), 270–275.
[12] Suzuki T., Sturgeon R.E., Zheng C, Hioki A., Nakazato T., Tao H.: *Anal. Sci.* **28** (2012), 807–811.
[13] Yang W., Gao Y., Wu L., Hou X., Zheng C. Zhu X.: *Microchim. Acta* **181** (2014), 197–204

Analytical aspects of model compounds toxicity assessment using MTT and Microtox bioanalytical assays

Błażej Kudłak[a], Zofia Mazerska[b], Jacek Namieśnik[a], Katarzyna Owczarek[a,*]

[a] *Gdansk University of Technology, Chemical Faculty, Department of Analytical Chemistry, Narutowicza 11/12 st., 80-233 Gdansk, Poland* ✉ *katowcza1@student.pg.gda.pl*
[b] *Gdansk University of Technology, Chemical Faculty, Department of Pharmaceutical Technology and Biochemistry, Narutowicza 11/12 st., 80-233 Gdansk, Poland*

Keywords
bioanalytics
bioassays
endocrine disrupting compounds
food contamination

Abstract
More and more increased intensity of anthropopressure processes can be observed, among other things, in the release of great quantities of synthetic substances into the environment, including the endocrine disrupting compounds. The effects of the above-mentioned human impact on the environment not only can be noticed in the homeostasis disturbance in ecosystems, but they are also visible with respect to human. The high incidence rate of cancer and other civilization diseases has become a serious social problem. Therefore, all attempts to broaden the knowledge about the relations and mechanisms triggering the development of relevant diseases are justified. The goal of the conducted research was to use bioanalytic methods to evaluate cytotoxicity and acute toxicity of the selected endocrine disrupting compounds which cause serious food contamination (bisphenol A, 4-nonynphenol, 4-*tert*-octylphenol, diethylstilbestrol, bisphenol A diglycidyl ether and its derivatives and phthalates) with MTT cel viability assay and Microtox®

1. Introduction

Endocrine disrupting compounds is a name for exogenous substances which, by disrupting the proper functioning of the endocrine system, cause undesired health effects on the organism exposed to them or on their offspring [1]. The mechanisms of the activity of those compounds may vary, starting from disruptions of the synthesis pathway of a given hormone, binding with another receptor to a direct modification of the activity of natural hormones. It should be noted that the signal regulation of the endocrine system takes place with the participation of a very low concentration of transmitters (hormones) and that is why an exposure of an organism to even small doses of endocrine active compounds may disrupt its homeostasis [2].

A vast number of chemical substances is considered to belong to the group of endocrine compounds, including substances which occur naturally in the environment, such as mycotoxins and phytoestrogens, and substances which have

been artificially released to the environment as a result of human activities (synthetic hormones, growth promoters, phthalates, bisphenols, metals, pharmaceutical residues, organochlorine compounds including PCBs) [2]. The chemicals selected for the studies were: bisphenol A (BPA), BPA diglycidyl ether (BADGE) and its derivatives, 4-*t*-octylphenol (4-t-OC), 4-nonylphenol (4NP), phthalates and diethylstilbestrol (DES). All of the aforementioned compounds are present in food products and their presence may be linked to numerous diseases affecting people and animals. They are mainly suspected to contribute to the induction of neoplastic diseases such as breast and prostate cancer, metabolic diseases, including obesity, genetic modifications and impairment of reproductive functions. They are also suspected to have mutagenic and cytotoxic effects and to disrupt regulatory pathways of some organs [1, 3–7].

The observed intensive development of the biological assessment methods for the levels of toxic substances in the environment results primarily from the fact of their high sensitivity in comparison to conventional chemical analysis procedures. These methods use advanced instrumental techniques both at the detection, identification and quantification stages. The analysis of biological response being the result of xenobiotic exposure, on the one hand allows to determine the risk of toxic effects resulting from the presence of a specific compound, and on the other hand, can provide the basis for the development of appropriate biological tests markers for the presence of toxic substances in a given environment. Presented study is focused on evaluation of cytotoxicity with MTT cell viability assay and acute toxicity with Microtox® assay. Measurement of the cell life processes activity changes is conducted on the cell line or on the microorganism treated with serial dilutions of selected chemical compound and compared to the control (without the chemical). The measurement is usually based on application of colorimetric, fluorimetric, bioluminescence or isotopic techniques. Toxicity is assessed on the basis of the IC_{50} value, defined as the concentration affecting 50% of cells compared to untreated control cells, Among the colorimetric techniques, the one most widely used quantitative method is MTT assay, developed in the 80s of 20th century [8]. The MTT assay is based on the measurement of mitochondrial metabolic activity by detection of the reduction of the soluble yellow MTT tetrazolium salt to purple MTT formazan by action of mitochondrial dehydrogenase [9]. Microtox® assay is based on bioluminescence of seawater bacteria *Vibrio fischeri*. It was developed in late 70s of 20th century [10]. It is used to evaluate acute toxicity of the sample which is estimated by bioluminescence intensity decrease. The designated parameter is effective concentration (EC_{50}) causing 50% decrease in bioluminescence.

The aim of this work was to assess cytotoxicity and acute toxicity of selected xenbiotics, that are common food and environmental contaminants using bioanalytical methods. These chemicals are linked to numerous diseases affecting humans thus the information about the concentrations, that may induce those diseases, is very important.

2. Experimental

2.1 Chemicals

MCF-7 human breast adenocarcinoma cell line, Minimum Essential Medium Eagle (MEM), trypsin-EDTA, Fetal Bovine Serum (FBS), Penicillin-Streptomycin, Thiazolyl Blue Tetrazolium Bromide (MTT), dimethylsulfoxide DMSO, ethanol (HPLC grade), bisphenol A, DES, BADGE and its derivatives, 4-*t*-octylphenol, 4-nonylphenol, dioctyl and diethyl phthalates were purchased from Sigma Adrich. Microtox assay kit was purchased from Omniwater (UK).

2.2 MTT assay for quantitative cytotoxicity determination

The MCF-7 cells were plated on 96-well plates at a density 8×10^3 cells per well and allowed to attach for 24 before treatment with xenobiotics. Stock solution of selected compounds (4, 2, 1, 0.5, 0.1, 0.05 mg/mL) were prepared by dissolving in absolute ethanol. The final concentration were obtained by appropriate dilution of the stock solutions in the culture medium. Cells were exposed to 0.5–40 μg/mL of each compound for 3, 6, 24 and 72 h. The final ethanol concentration in the medium was 1% for each sample. After each incubation time cells were washed with 200 μL of fresh culture medium. 50 μL of MTT dissolved in deionized water (4 mg/mL) was added to each well and cells were incubated for 4 h. After the end of incubation period the media were discarded and 75 μL of DMSO was added to dissolve formazan crystals. Plates were shaken for 20 min and after that time the absorbance was measured at 550 nm using a spectrophotometer (Tecan Infinite® 200 PRO microplate reader). All experiments were performed in triplicate and all cells used in the experiment were between 5 and 15 passage.

2.3 Microtox assay for acute toxicity assessment

Gram-negative bacteria *Vibrio fischeri* were treated with model solutions of selected xenobiotics The exposure time was 30 min. The bacteria was purchased in freeze-dried form and activated by rehydratation with a reconstitution solution (specially prepared nontoxic Ultra-Pure Water) to provide a ready-to-use suspension of organisms. The osmolarity of all standard and samples was adjusted to 2% NaCl for the optimal performance. A dilution series of the samples to be analysed were prepared in 2% NaCl. Stock solution of selected compounds (4, 2, 1, 0.5, 0.1, 0.05 mg/mL) were prepared by dissolving in absolute ethanol. The final concentration were obtained by appropriate dilution of the stock solutions in the distilled water (final ethanol concentration was 2%). A fixed amount of bacteria was added to the dilution vials. The light emission of this bacteria in contact with different samples and exposure times was measured using the Microtox 500 analyser. The data were processed according to the Basic Test Protocol.

3. Results and discussion

3.1 MTT assay

The results obtained during studies of influence of different concentrations of selected chemicals that belong to the endocrine disrupting compounds group to MCF-7 cells determined by MTT cytotoxicity assay are shown on Fig. 1. The results are demonstrated in dose-dependent significant cytotoxic effect. In most cases it was not possible to determine IC_{50} value, because most of examined chemicals did not caused such cell viability decrease in studied concentrations range. To compare the results, the areas under each chart was calculated and then presented on the cumulative chart (Fig. 2). It is easy to see, that the compound that causes the highest viability inhibition in every incubation time for concentrations 4–2 mg/mL is 4-*t*-octylphenol. Also occurrence of a chemical hormesis can be observed, especially for BPA (3 h and 6 h incubation) and diethyl phthalate (24 h incubation). It is probably connected with strong estrogenic activity of these compounds, which stimulate growth processes of neoplastic cells of breast adenocarcinoma by influencing estrogen receptors present in these cells. Chemical hormesis phenomenon also can be caused by enzymatic activity of mitochondrial dehydrogenases reducing MTT reagent, that are still active for some time since cell death. Strong concentration dependence could be also observed, with highest values of viability inhibition for highest concentrations and only slight changes(comparing to control) for lowest ones.

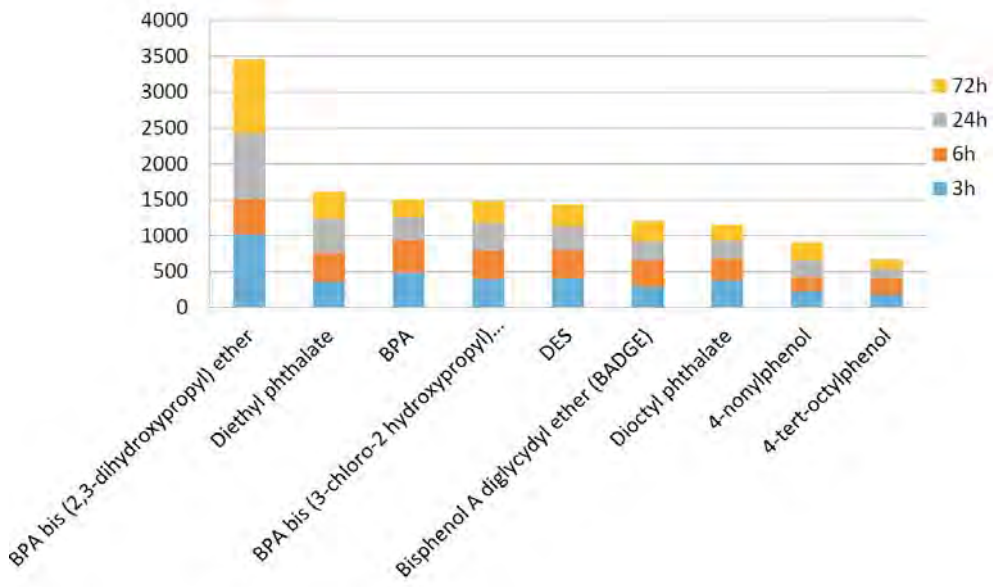


Fig. 2. Cumulative chart comparing toxicity of selected xenbiotics.

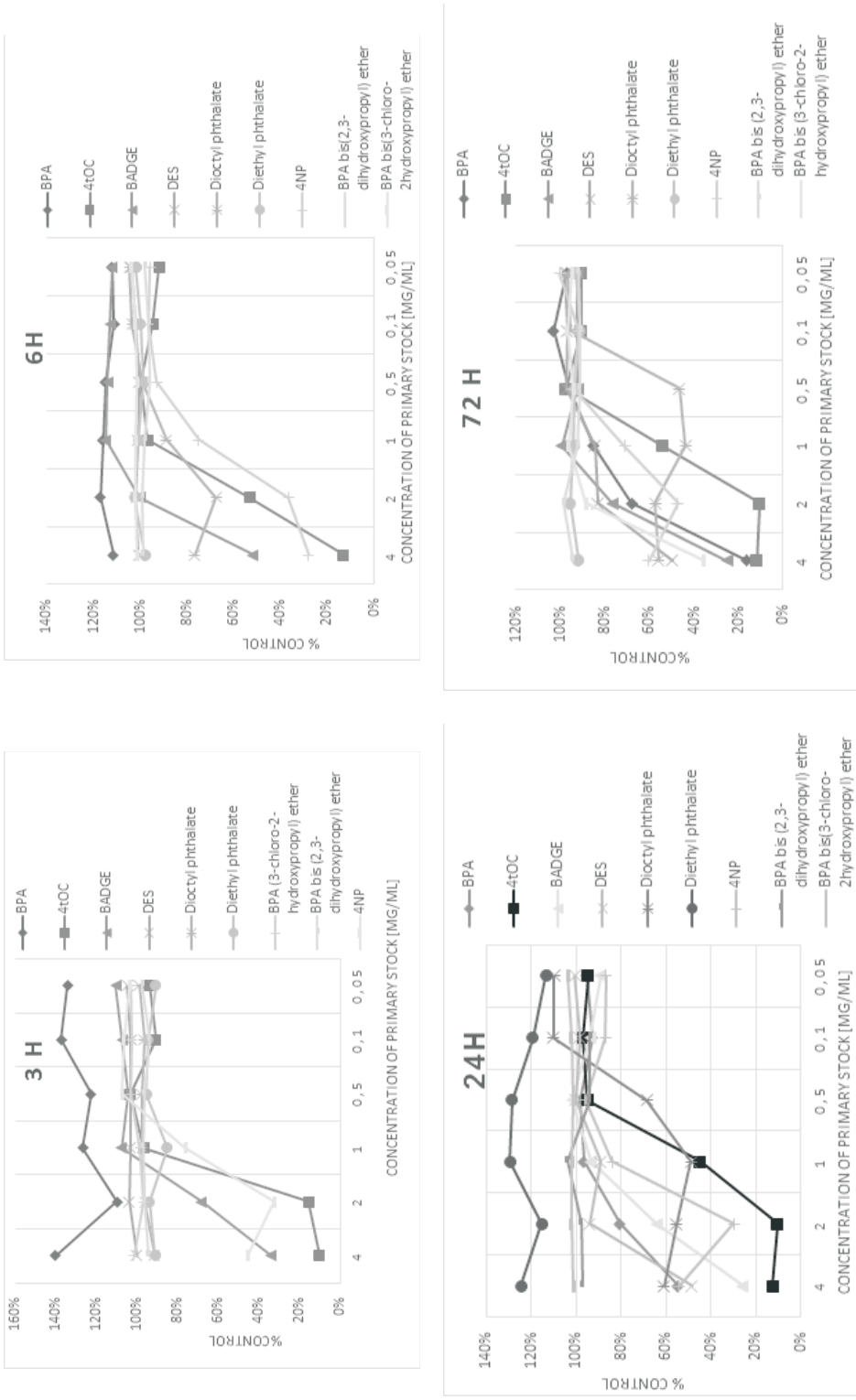


Fig. 1. Cytotoxic effect in concentration-dependent manner.

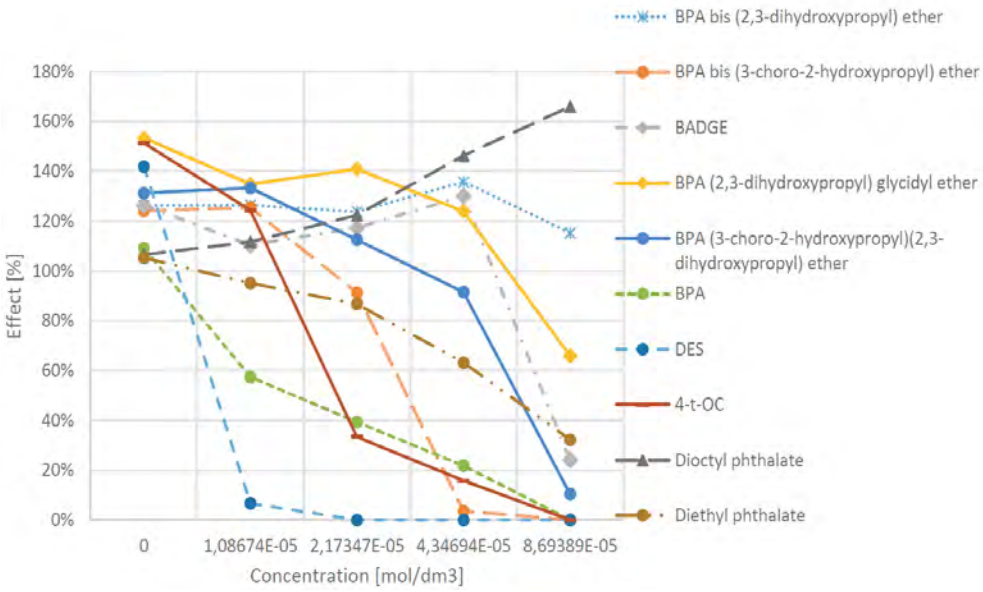


Fig. 3. Toxicity of selected xenobitics toward *Vibrio fischeri*.

Table 1
EC_{50} values calculated for selected compounds.

Compound	EC_{50} [μmol/dm^3]
BPA bis(3-chloro-2-hydroxypropyl)ether	40.2
Bisphenol A	46.7
BPA bis (3-chloro-2-hydroxypropyl)(2,3-dihyroxypropyl)ether	61.3
4-t-octylphenol	72.0
Bispehol A diglycidyl ether (BADGE)	89.2
Diethylphthalate	108.0
BPA (2,3-dihydroxypropyl)glycidyl ether	115.1
BPA bis (2,3)-dihydroxyproyl ether	–7.2

3.2 Microtox assay

Acute toxicity was assessed with Microtox assay. Obtained results and calculated EC_{50} values are shown in Fig. 3 and Table 1. Calculating EC_{50} value for diethylstilbestrol and 4-nonylphenol was impossible in the studied concentration range (highly toxic). The highest acute toxicity towards Vibrio fischeri bacterium was assessed for BPA bis (3-chloro-2-hydroxypropyl) ether and bisphenol A with the lowest EC_{50} value (40.2 and 46.7 μmol/dm^3). Chemical hormesis can be observed for most of the lowest compounds concentrations, despite BPA and diethyl

phthalate. Dioctyl phthalate seems to induce bacterial luminescence (as shown in the Fig. 3 and Table 1).

4. Conclusions

Cytotoxicity and acute toxicity of selected compounds, that are suspected to have endocrine disrupting effect toward living organisms, was assessed by two aforementioned bioassays MTT cell viability assay and Microtox assay. Obtained results does not seem to be correlated, although in both cases 4-nonylphenol and 4-*t*-octylphenol show the highest toxicity. The lack of correlation results from the differences in test organisms and the principle of the assays. BPA bis-(2,3-dihydroxypropyl ether) shows the lowest toxic effect both towards MCF-7 cells and *Vibrio fischeri* bacteria. Hormesis phenomenon observed in MTT test for bisphenol A and diethyl phthalate after short incubation period may indicate an estrogenic activity of these chemicals.

References

[1] Hampl R., Kubatova J., Starka J.: *J. Steroid Biochem. Mol. Biol.*, in press, DOI: 10.1016/j.jsbmb.2014.04.013
[2] Kudłak B., Szczepańska N., Owczarek K., Mazerska Z., Namieśnik J.: *Crit. Rev. Anal. Chem.* **45** (2015), 191–200.
[3] Kudłak B., Szczepańska N., Owczarek K., Mazerska Z., Namieśnik J.: Endocrine disrupting compounds. Problems and challenges. In: *Emerging Pollutants in the Environment. Current and Further Implications*. M.L. Larramendy (edit.). Rijeka, InTech 2015.
[4] Michałowicz J.: *Environ. Toxicol. Pharmacol.* **37** (2014), 738–758.
[5] Valenta H.: *J. Chromatogr. A* **815** (1998), 75–92.
[6] Tatay E., Meca G., Font G., Ruiz M.-J.: *Toxicol. In Vitro* **28** (2014), 95–103.
[7] Rochester J.R.: *Reprod. Toxicol.* **42** (2013), 132–155.
[8] Mosmann T.: *J. Immunol Methods* **65** (1983), 55–63
[9] Radosevic K., Tonkovic T., Slivac I., Kniewald Z., Srcek V.G.: *Bull. Environ. Contam. Toxicol*. **86** (2011), 252–257.
[10] Wilk P., Szalińska E.: *Technical Transactions* **108** (2011), 247–263.

Arctic as a reservoir for polycyclic aromatic hydrocarbons and polychlorinated biphenyls – water samples from Foxfonna glacier

Aneta Pacyna*, Żaneta Polkowska

Gdańsk University of Technology, Chemical Faculty, Department of Analytical Chemistry, 11/12 Narutowicza st, 80-233 Gdańsk, Poland ✉ an.pacyna@o2.pl

Keywords
arctic
glaciers
long range pollutant transport
polychlorinated biphenyls
polycyclic aromatic hydrocarbons

Abstract
Polycyclic aromatic hydrocarbons and polychlorinated biphenyls are groups of stable compounds, hard to biodegrade, potentially toxic. Their presence in Arctic environment may have a big impact on natural balance, fauna and flora. Although Arctic areas should be unpolluted, some amounts of pollutants are transferred from lower latitudes with sea currents and air masses. This paper presents the amounts of selected persistent organic pollutants detected in water samples from Svalbard

1. Introduction

Due to harsh climate conditions and characteristic landform Arctic areas were little changed by humans. Although there are local sources of pollution (like cruise ships, drilling platforms, wastes from town), bigger impact on increased pollutants concentration has long range transport of pollutants from lower latitudes (Europe, Russia, Canada). Migration of pollutants is facilitated by specific arrangement of air masses during winter and early spring. Also because natural regeneration processes are slow, contamination caused by anthropogenic activities has a significant impact on the quality of the environment. The introduction of compounds, like heavy metals or persistent organic pollutants may disrupt natural homeostatic mechanisms which in turn may lead to the collapse of ecological balance. Persistent organic pollutants may derived from many various sources, like from fossil fuel burning, forest fires, industry activity etc. Polycyclic aromatic hydrocarbons and polychlorinated biphenyls belong to this group of compounds and their main properties are presented on Fig. 1.

Recently a growing interest is noted on the effects of especially long-term health effects of pollution on living organisms. PCB and PAH may bioaccumulate in fatty tissue and their toxic effect was proved in many research study [1–4]. Topographic analysis of glaciers in Svalbard has shown that since 1936 they reduced each year, resulting in a reversion substantial part of them [6–8] and when snow melts impurities contained in it are also released.

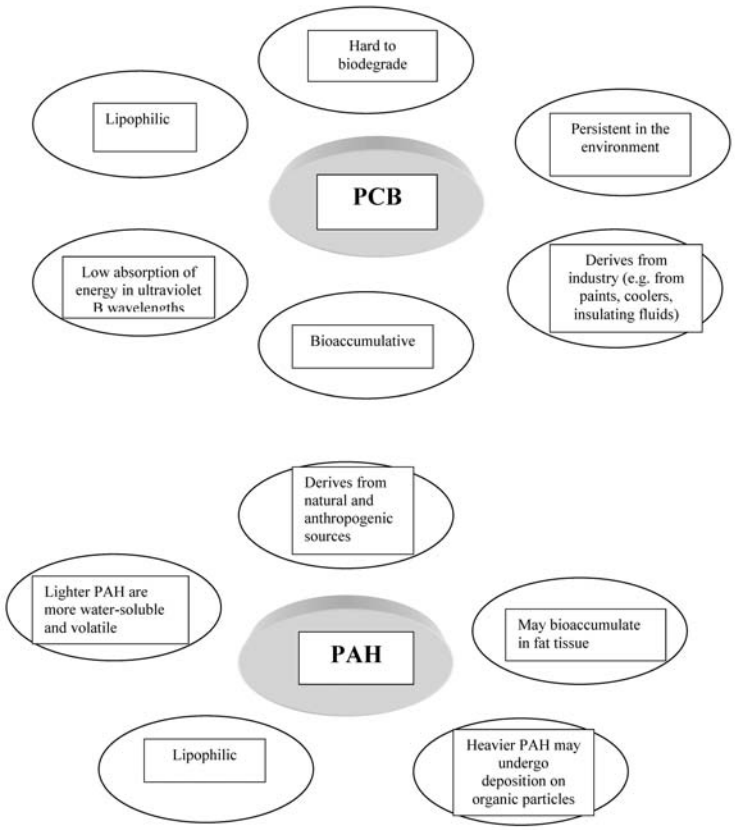


Fig. 1. Main characteristic of PAHs and PCBs.

2. Experimental

2.1 Sampling

23 samples of surface water were collected by from Foxfonna glacier between July and August 2012. Samples were transferred into bottles and transported by ship into laboratory for further analysis (at 4 °C). Internal standards were added into 500 ml of each sample. Next 15 ml of dichloromethane (DCM) was added and samples were shaken for 15 minutes. Extract was collected and procedure was repeated. In a next step sam-ples were evaporated into 300 µl under gentle nitrogen stream and stored in the refrigerator. All analysis in experimental part were done in Department of Analytical Chemistry (Gdańsk University of Technology). Internal standards contains the mixture of compounds from the group of PCBs in isooctane having a concentration of each compound of 5 µg/ml (PCB 28, 138, 153, 101, 118, 180) and mixture of 16 compounds from the group of PAH in DCM having a concentration of each compound 2000 µg/ml. Fig. 2 presents the extent of Foxfonna glacier.

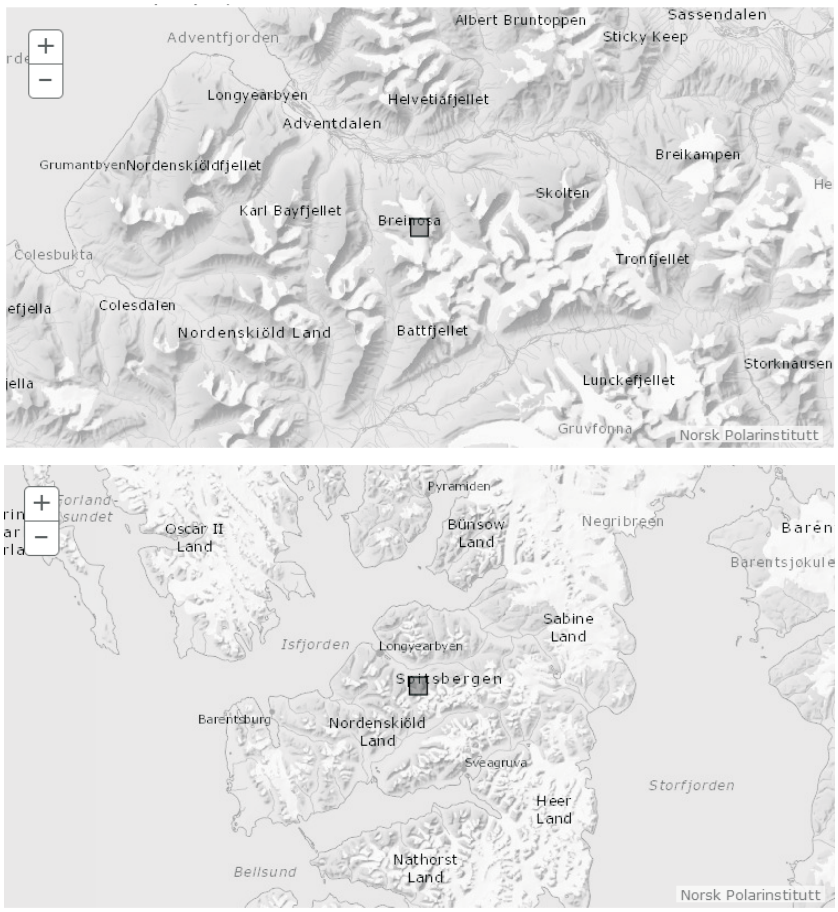


Fig. 2. Extent of Foxfonna glacier [9].

2.2 Instrumentation

Liquid-liquid extraction was used for sample preparation. Final determination technique for all PCBs and PAHs analysis was gas chromatography-mass spectrometer (GC: Agilent Technologies 7890A System; MS: Agilent Technologies 5975C Inert MSD). All analysis were done in the second quarter of 2014.

3. Results and discussion

Analysis confirms presence of 14 PAHs and 3 PCB congeners. The highest average value was detected for acenaphtene (about 17 μg/l), pyrene (about 5.1 μg/l), acenaphtylene (2.5 μg/l), phenanthrene (2.3 μg/l) and naphthalene (1.7 μg/l). The presence of chrysene and dibenzo[*a,h*]anthracene was not confirmed (levels were below limit of detection). In case of PCB only PCB 28, 118 and 153 were

detected. The highest average value was for PCB 118 (about 0.012 μg/l), PCB 28 and PCB 153 were at lower levels (average value was about 0.003 μg/l). PCB 101, 138 and 180 were below limit of detection in each samples. Table 1 and 2 are presenting levels of analysed compounds.

Polycyclic aromatic hydrocarbons may derived from many various sources both natural (volcanic activity, forest fires, microbial activity in plant detritus) and anthropogenic (fossil fuel burning, emissions from shipping, flaring at offshore oil installations, oil seeps). Another source might be oil seeps from oil reservoirs or hydrocarbon source rocks. PCB comes mostly from industry, but because of high toxicity production is banned in many countries and usage is under strict control (e.g. plasticizers in paints, coolants and insulating fluids for transformers). Although usage of those compounds decreased, some amounts are still present in natural environment (due to high stability and their chemical inability to be oxidized and reduced in the natural environment).

Table 1
Level of determined polycyclic aromatic hydrocarbons.

Compound	Range [ng/l]	Average [ng/l]	Standard deviation
Naphtalene	176–13200	1747	2705
Acenaphthylene	57.3–7643	2535	1944
Acenaphthene	406–57483	17041	14051
Fluorene	8.30–1633	321	369
Phenanthrene	49.6–10490	2311	2592
Anthracene	1.60–614	302	172
Fluoranthene	0.70–373	85	89
Pyrene	189–12646	5104	3120
Benzo[*a*]anthracene	2.30–326	53	81
Chrysene	*n.d.*	*n.d.*	*n.d.*
Benzo[*b*]fluoranthene	31.2–317	98	61
Benzo[*k*]fluoranthene	31.2–317	98	61
Benzo[*a*]pyrene	5.64–2372	682	449
Indeno[1,2,3-*cd*]pyrene	1.56- 10.8	6	2
Dibenzo[*a,h*]anthracene	*n.d.*	*n.d.*	*n.d.*
Benzo[*g,h,i*]perylene	138–138	138	26

Table 2
Level of determined polychlorinated biphenyls.

Compound	Range [ng/l]	Average [ng/l]	Standard deviation
PCB 28	0.60–6.13	2.89	1.84
PCB 118	2.43–30.1	12.4	5.72
PCB 153	2.45–3.14	2.80	0.74

4. Conclusions

Arctic areas should be an unpolluted place, but mostly because of human activity many toxic compounds enters this unique ecosystem. Pollutants may reach this region with sea current or air masses and most of them are transported from lower, more urbanized latitudes. Because of harsh climate conditions natural regeneration processes are slow and pollutants might accumulate in various components of the ecosystem. Results suggest need for further monitoring of Arctic areas.

References

[1] Ruman M., Kozak K., Lehmann S., Kozioł K., Polkowska Ż.: *Ecol. Chem. Eng. S* **19** (2012), 571–584.
[2] Letcher R.J., Bustnes J.O., Dietz R., Jenssen B.M., Jorgensen E.H., Sonne C., Verreault J., Vijayan M.M., Gabrielsen G.W.: *Sci. Total Environ.* **408** (2010), 2995–3043.
[3] Hoffman D.J., Rice C.P., Kubiak T.J.: PCBs and dioxins in birds. In: *Environmental Contaminants in Wildlife*. *Interpreting Tissue Concentrations*. W.N. Beyer, G.H. Heinz, A.W. Redmon-Norwood (edits.). CRC Press, Boca Raton 1996, p. 165–208.
[4] Bogan J.A., Bourne W.R.P.: *Nature* **240** (1972), 358.
[5] Kohler J., Nordli P.Ř., Brandt O., Isaksson E., Pohjola V., Martma T., Aas H.F.: *Svalbard Temperature and Precipitation, Late 19th Century to the Present.* ACIA Final Report 2013.
[6] Kohler J., James T.D., Murray T., Nuth C., Brandt O., Barrand N.E., Aas H.F., Luckman A.: *Geophys. Res. Lett.* **34** (2007), article number L18502.
[7] LafflyD., Mercier D.: *Int. J. Remote Sens.* **23** (2002), 4743–4760.
[8] Nuth C., Kohler J., Aas H.F., Brandt O., Hagen J.O.: *Ann. Glaciol.* **46** (2007), 106–116.
[9] http://stadnamn.npolar.no/ (accessed 17 June, 2015)

Electrochemical hydride generation of tellurium hydride coupled with QT AAS

TINA RESSLEROVÁ*, JAKUB HRANÍČEK

Department of Analytical Chemistry, Faculty of Science, Charles University in Prague, Hlavova 8, 128 43 Prague 2, Czech Republic ✉ *tina.resslerova@natur.cuni.cz*

Keywords
AAS
electrochemical hydride generation
interference
tellurium hydride

Abstract
Hydride generation is a method of sample introduction commonly used in atomic spectrometry for the determination of elements forming volatile hydrides. It is a suitable derivatization technique for important environmental pollutants like heavy metals. Electrochemical hydride generation is a more ecological and economical alternative, avoiding the use of tetrahydridoboritanes. Its drawback is, that some elements are not so easily transferred into the volatile compound. One of these is tellurium and this work is focused on creating a suitable method for its determination. The generation conditions were optimised and figures of merit were determined for the optimal conditions. The effect of selected elements from various element groups was also tested, including other hydride forming elements, transition metals and metals from IA and IIA groups.

1. Introduction

Hydride generation is a method of sample introduction commonly used in atomic spectrometry for the determination of elements forming volatile hydrides. It possesses several advantages like the separation of the analyte from the matrix and thus reduction of the majority of interferences, and increase of determination sensitivity [1]. Hydride generation is a suitable derivatization technique for important environmental pollutants like mercury, lead, and arsenic.

Electrochemical hydride generation is a more ecological and economical approach to hydride generation, avoiding the necessity to use tetrahydridoboritanes, which are expensive and unstable. [2] However, hydrides of some elements are quite hard to obtain.

Tellurium is a rare element, but its use in industry is rising for example in fotovoltaics the CdTe alloy is used [3] and it is used also in CD/bluray production [4]. This leads to its possible accumulation in the environment and necessity to develop sensitive determination techniques.

Tellurium chemical hydride generation is a well-established method. [5, 6] This work aims to develop an alternative method of electrochemical generation of tellurium hydride for the use in atomic absorption spectroscopy.

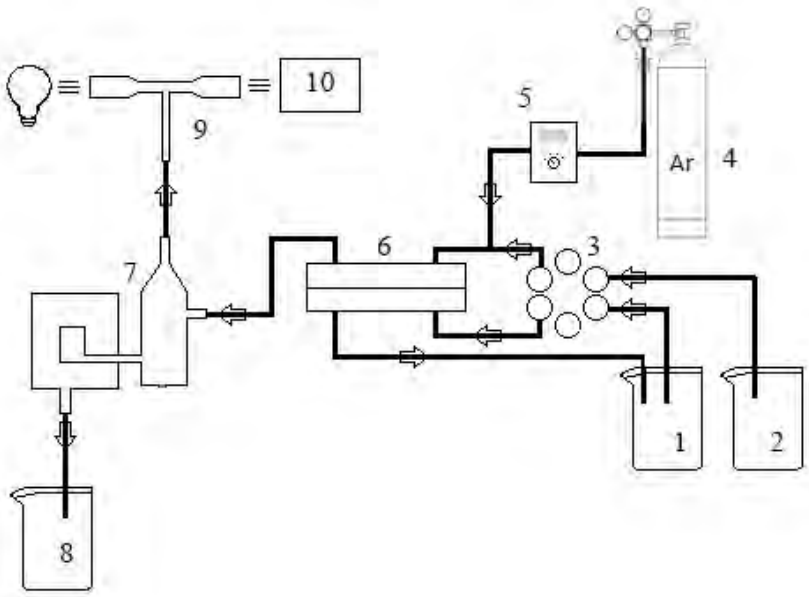


Fig. 1. Experimental setup: (1) anolyte, (2) catholyte, (3) peristaltic pump, (4) carrier gas, (5) gas flow controller, (6) electrolytic cell, (7) gas/liguid separator, (8) waste, (9) quartz tube atomizer, (10) detector.

2. Experimental

2.1 Reagents and chemicals

Sulfuric acid of the suprapure purity (96 %, Merck) was used for the preparation of electrolytes. Stock solutions of bismuth and nickel (1.001±0.005 g l^{-1}, Merck), stock solutions of tellurium, arsenic, selenium, lead, tin, antimony and zinc (1.000±0.002 g l^{-1}, Analytika, Prague) and solid $CaCl_2.6H_2O$, $MgCl_2.6H_2O$, KCl and NaCl p.a. purity (Lachner, CZ) were used for preparation of analysed samples. Deionised water from Milli QPLUS (Millipore, USA) was used for dilution of solutins. Argon of 99.998% purity (Linde Technoplyn, Prague) was used as carrier gas. A 5 cm long, 0.5 mm wide platinum wire with purity 99.99% (Safina, CZ) was used as the anode and a 1 mm wide wire of lead with purity 99.99 % (Aldrich)of the same length was used as the cathode.

2.2 Instrumentation

A Pye Unicam 939 AA Spectrometer (Unicam, England) was used for the experiments together with a laboratory made hydride generation apparatus (Fig. 1). The catholyte and anolyte flow was driven by a peristaltic pump. The sample and carrier gas were injected into the catholyte flow. Then both

electrolytes entered the electrolytic cell where the hydride was generated. The anolyte was recycled and catholyte was separated from the gaseous products of the reaction in a separator.

3. Results and discussion

The optimal working conditions for tellurium hydride generation were as following: The flow of electrolytes was 3 ml min^{-1}, the flow of carrier gas was 80 ml min^{-1}, the current was 1 A to which corresponded 10 V. The concentration of anolyte was 2 mol l^{-1} and of catholyte 0.5 mol l^{-1}. For these conditions the figures of merit were found, the limit of detection was found to be 0.2 mg l^{-1}, with limit of linearity 20 mg l^{-1} and a repeatability of 6.7%.

The interference study covered other hydride forming elements (As, Se, Bi, Sn, Sb, and Pb), transition metals (Fe, Ni, Cu, and Zn) and alkaline and alkali-earth metals (K, Ca, Na, and Mg). The hydride forming elements interfered when in ratio 1:10 (Te:interferent) which was most probably caused by competition between tellurium and the interferent in both generation and atomization steps. The transition metals interfere in aqueous phase and by more of less permanently modifying the electrode. Interference of transition metals was also studied in detection of selenium [7] where the results were similar; however the interference was studied only for ratios larger than 1:1. In small concentrations most of these elements worked as a reaction modifier by increasing the signal even by 50%. The alkaline and alkali-earth metals interfered significantly only in great excess (1:1000 and more).

The accuracy of the method was tested on several spiked beverage samples (sweetened and unsweetened bottled water, tea and red wine).

4. Conclusions

The optimal working conditions for tellurium hydride generation were found and the limit of detection was found to be 0.2 mg l^{-1}. The interference study was done and and the transition metals were found to be the most important group of interferents, because of the electrode modification and modification of reactions in the cell. The results obtained from spiked samples were mostly in agreement with expected values.

Acknowledgments

We would like to thank to GAČR P206/14-23532S for funding.

References

[1] Weltz B., Sperling M.: *Atomic Absorption Spectrometry.* 3rd Ed. Weinheim, Wiley-VCH 1999.
[2] Červený V., Rychlovský P., Hraníček J., Šíma J.: *Chem. Listy* **103** (2009), 652–660.
[3] *Handbook on the Toxicology of Metals.* 3rd ed. Gerhardsson L., Nordberg G.F., Fowler B.A., Nordberg M., Friberg L.T. (edits). Amsterdam, Elsevier 2007.

[4] Kolobov A. V., Fons P., Frenkel A.I.: *Nat. Mater.* **3** (2004), 703–708.
[5] Rigby C., Brindle I.D.: *J. Anal. At. Spectrom.* **14** (1999), 253–258.
[6] Grotti M., Lagomarsino C., Frache R.: *J. Anal. At. Spectrom.* **20** (2005), 1365–1373.
[7] Bolea E., Laborda F., Belarra M.A., Castillo J.R.: *Spec. Chim. Acta B* **56** (2001), 2347–2360.

Anodic voltammetric determination of 2-nitrophenol at a carbon film electrode in model samples of drinking water

TEREZA RUMLOVÁ*, JIŘÍ BAREK

Charles University in Prague, Faculty of Science, Department of Analytical Chemistry, UNESCO Laboratory of Environmental Electrochemistry, Hlavova 8, 128 43 Prague, Czech Republic
✉ *rumlova@natur.cuni.cz*

Keywords
carbon film electrode
differential pulse voltammetry
drinking water
2-nitrophenol

Abstract
This work is focused on the development of new differential pulse voltammetric (DPV) method for the determination of submicromolar concentrations of 2-nitrophenol using carbon film electrode. The highest and the best developed DPV peak was obtained using Britton-Robinson buffer of pH = 6.0. The best repeatability was obtained by inserting regeneration potentials E_{in} = 1300 mV; E_{fin} = 0 mV. The calibration curves of 2-nitrophenol in both deionized and drinking water were linear in the concentration range from 4×10^{-7} to 1×10^{-4} mol L^{-1}. Limit of quantification for DPV in deionized water was 2.0×10^{-7} mol L^{-1} and in drinking water 1.0×10^{-7} mol L^{-1}.

1. Introduction

Nitrophenols are well known environmental pollutants, which come mainly from industrial processes, especially from fabrication of common pesticides, pharmaceuticals, explosive and dyes [1]. Furthermore, they are often used as plants growth stimulators [2]. Nitrophenols are suspected from carcinogenic, mutagenic and teratogenic effects [3] and many of them are listed in EPA Priority Pollutants List [4]. They are easily electrochemically oxidisable and reducible as proved by electrochemical study of 4-nitrophenol at glassy carbon electrode as a model to understand the quantification of hydroxyl and nitro derivatives of aromatic compounds [5].

Carbon film electrode is any solid electrode on which the carbon film is deposited. For this work, polished silver solid amalgam electrode has been used as a conductive substrate. Carbon film electrode was developed as an alternative to commercially available screen-printed carbon electrodes compatible with the concept of "green analytical chemistry" [6]. The biggest advantage of carbon film electrode is its simple, fast and inexpensive fabrication. When necessary, the film can be easily removed by wiping it off by a filter paper and a new film can be created by immersing the electrode's surface into the carbon ink which is

prepared from a carbon powder homogenously mixed with polystyrene and dispersed in a suitable volatile solvent. Another advantage is its wide potential window (ca. 3 V span), high sensitivity, and relatively low noise of measurements.

Using modern voltammetric methods the oxidisable organic compounds can be determined at carbon film electrode as demonstrated by the determination of selected drugs (e. g. paracetamol [7] and guanine and adenine [8]).

2. Experimental

2.1 Reagents and chemicals

The stock solution of 2-nitrophenol (1×10^{-3} mol L^{-1}) was prepared by dissolving of 0.0139 g of the substance (Sigma-Aldrich, CAS reg. Number [88-75-5]) in deionized water and filling up to 100 mL. Boric acid, phosphoric acid, acetic acid, sodium hydroxide and potassium chloride, were of p.a. purity (Lachner, Czech Republic). Britton Robinson buffer was prepared by mixing of 0.2 mol L^{-1} sodium hydroxide with the mixture of 0.04 mol L^{-1} of boric, phosphoric, and acetic acid. The pH was measured by pH meter Jenway with combined glass electrode (type 924005, Jenway, UK) and deionized water (Millipore Q plus system, Millipore, USA) for preparation of all solutions was used.

2.2 Instrumentation

All measurements were carried out using μAutolab type III potenciostat (Metrohm Autolab, Netherland) and the software (NOVA 1.10, Metrohm Autolab, Netherland) under the operating system Microsoft Windows 8 (Mircrosoft Corp., USA). All measurements were carried out in a three-electrode system with platinum wire (Elektrochemicke detektory, Czech Republic) auxiliary electrode, silver/silver chloride reference electrode (3 mol L^{-1} KCl, Elektrochemicke detektory, Czech Republic) and carbon film electrode on polished silver solid amalgam electrode substrate, disk diameter 0.413 mm (EcoTrend Plus, Czech Republic) as working electrode. The scan rate 20 mV s^{-1}, the pulse amplitude 50 mV, and pulse width 100 ms were used.

The conductive carbon ink for modification of the electrode surface was prepared by mixing 0.01 g of polystyrene, 0.09 g of carbon powder (microcrystalline graphite 2 μm, CR 2, Maziva Tyn, Czech Republic) and 0.5 mL of 1,2-dichloroethane (MERCK, Germany). This mixture was homogenized by intensive agitation for 3 minutes at Lab dancer (IKA®-Werke GmbH & Co. KG, Germany).

3. Results and discussion

At first the influence of pH (2–12) on DPV behavior of 2-nitrophenol (1×10^{-4} mol L^{-1}) has been tested. The highest and the best developed peak was obtained using Britton-Robinson buffer of pH = 6.0.

Afterwards, the possibility of inserting the regeneration potential cycles to diminish negative influence of the electrode passivation was investigated. These regeneration potential cycles (initial potential (E_{in}) before and final potential (E_{fin}) after the peak or both initial and final potential before the peak) were imposed on the electrode in 150 cycles, with frequency 10 cycles per second. The best repeatable and the best developed peaks were obtained using regeneration potential cycles between E_{in} = 1300 mV, E_{fin} = 0 mV.

All above mentioned conditions obtained during optimization processes were used for measuring DPV calibration dependences of 2-nitrophenol in deionized water and in model samples of drinking water. Britton-Robinson buffer of pH = 6.0 was used as supporting electrolyte and linear calibration curves were obtained in the concentration range from 4×10^{-7} mol L^{-1} to 1×10^{-4} mol L^{-1}.

The applicability of the developed DPV method was tested on the direct determination of 2-nitrophenol in model samples of drinking water. The model samples of 2-nitrophenol were prepared by mixing 9.0 mL of tap water sample containing the exact amount of 2-nitrophenol stock solution and 1.0 mL of the Britton-Robinson buffer of pH = 6.0. The concentration range from 2×10^{-7} mol L^{-1} to 1×10^{-4} mol L^{-1} was measured. The DP voltammograms of 2-nitrophenol in drinking water are depicted in Fig. 1.

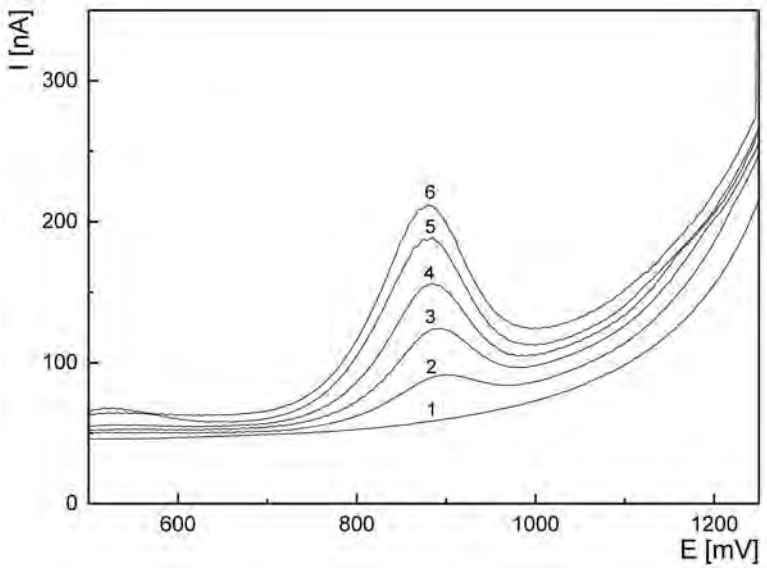


Fig. 1. DP voltammograms of 2-nitrophenol at carbon film electrode in drinking water model samples (9 mL of spiked drinking water was filled up to 10 mL with Britton-Robinson buffer of pH = 6.0). The curves correspond to the concentrations of 2-nitrophenol in 9 mL of drinking water model sample: 0 (1); 2 (2); 4 (3); 6 (4); 8 (5); and 10 (6) μmol L^{-1}.

4. Conclusions

The DPV method for determination of 2-nitrophenol using carbon film electrode based on anodic oxidation of the present hydroxyl group was developed. The best results were obtained using Britton-Robinson buffer of pH = 6.0 with optimum regeneration potentials E_{in} = 1300 mV, E_{fin} = 0 mV. The linear calibration dependences of 2-nitrophenol in deionized water with the limit of quantification 2.0×10^{-7} mol L^{-1} were obtained. The practical applicability of this newly developed DPV method, for direct determination of 2-nitrophenol at carbon film electrode in drinking water has been verified. Limit of quantification in model samples of drinking water, was $LOQ = 1.0\times10^{-7}$ mol L^{-1}.

Acknowledgments

This research was carried out in the framework of the Specific University Research (SVV 260205). JB thanks the Grant Agency of the Czech Republic (Project P206/12/G151) and TR thanks the Grant Agency of Charles University (Project GAUK 468214) for financial support.

References

[1] Asadpour-Zeynali K., Najafi-Marandi P.: *Electroanalysis* **23** (2011), 2241–2247.
[2] SPA, *List of Authorized Plant Protection Products and other means of Plant Protection*, Praha 2011.
[3] Fischer J., Vanourkova L., Danhel A., Vyskocil V., Cizek K., Barek J., Peckova K., Yosypchuk B., Navratil T.: *Int. J. Electrochem. Sci.* **2** (2007), 226–234.
[4] www.epa.gov, (accessed 27th January, 2015)
[5] Pfeifer R., Tamiasso Martinhon P. Sousa C., Moreira J. C., Chaer do Nascimneto M.A., Barek J.: *Int. J. Electrochem. Sci.*, in press.
[6] Yosypchuk B., Barek J., Fojta M.: *Electroanalysis* **18** (2006), 1126–1130.
[7] Khaskheli A.R., Fischer J., Barek J., Vyskocil V., Sirajuddin, Bhanger M.I.: *Electrochim. Acta* **101** (2013), 238–242.
[8] Seddon B.J., Osborne M.D., Lagger G., Dryfe R.A.W., Loyall U., Schafer H., Girault H.H.: *Electro-chim. Acta* **42** (1997), 1883–1894.

Comparison of HPLC and UPLC methods for the determination of melatonin in rice

WIDIASTUTI SETYANINGSIH[a, b, *], MIGUEL PALMA[b], CARMELO G. BARROSO[b]

[a] *Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Gadjah Mada University, Jalan Flora, Bulaksumur, 55281, Yogyakarta, Indonesia*
✉ *widiastuti.setyaningsih@ugm.ac.id*

[b] *Department of Analytical Chemistry, Faculty of Sciences, University of Cadiz, Campus de Excelencia Internacional Agroalimentario (CeiA3), Campus del Rio San Pedro, 11510, Puerto Real, Cadiz, Spain*

Keywords
HPLC
melatonin
rice
UPLC

Abstract
This study compares the HPLC and UPLC methods for the determination of melatonin in rice extracts. The methods were validated in terms of linearity, precision, limits of detection (*LOD*) and quantification (*LOQ*). Repeatability and intermediate precisions expressed as coefficient of variation (*CV*) were less than 2.5% for HPLC and 1.5% for UPLC. Linear regression models fit perfectly the standard curves with $R^2 > 0.99$ for both methods. The *LOD*s were 1.15 μg L^{-1} and 0.73 μg L^{-1} while *LOQ*s were 3.84 μg L^{-1} and 2.19 μg L^{-1}, for HPLC and UPLC respectively. High efficiency UPLC gradient is feasible and beneficial to substitute the conventional analysis of melatonin in rice grain by HPLC as permitted the analysis run time to be reduced by two-thirds thus consumes significantly less eluents.

1. Introduction

There is a growing interest in separation science to achieve dramatic increases in resolution, speed and sensitivity in liquid chromatography. Significant advances in technology were made for ultra-fast separation with high efficiency through an Ultra-Performance Liquid Chromatography (UPLC® Technology) by Waters [1].

UPLC has overcame the negative aspect of packed columns used in high-performance liquid chromatography (HPLC) by precisely delivering mobile phase at pressures up to 15,000 psi, thus enabling columns packed with smaller particles (sub-2 μm) to provide higher level of chromatographic performance [2]. Besides, because of the separation methods are significantly faster, UPLC technology reduces the use of eluents and wastes compared with conventional HPLC [3]. Furthermore, the UPLC system facilitates a low limit of detection since the signal--to-noise ratio is improved and the injection volume can be considerably reduced without losing the sensitivity [4].

In order to compare the performance of HPLC and UPLC, analyses for the determination of melatonin (*N*-acetyl-3-(2-aminoethyl)-5-methoxyindole; Fig. 1)

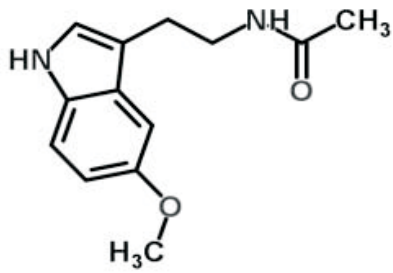


Fig. 1. Chemical structure of melatonin.

using both methods were studied. Research into melatonin continued with many results revealing the utility of this compound. Melatonin diminishes neurodegenerative diseases, such as Parkinson and Alzheimer [5]; it also acts as an anticancer agent [6]. Recently, research articles have appeared discussing methods of analysis of melatonin in different matrices; these methods include HPLC-EC [7, 8], HPLC-UV [9–11], HPLC-MS [12], ELISA [13–15], and GC-MS [16].

Although melatonin determination in different matrices has been reported, limited studies performed on separation of melatonin by UPLC, particularly in conjunction with fluorescence detection (FD). The aim of this study was to develop a new separation method by UPLC-FD and to compare with conventional HPLC for analysing melatonin in rice grain extracts.

2. Experimental

2.1 Reagents and chemicals

HPLC grade methanol, acetonitrile, and acetic acid were purchased from Merck.. Melatonin standard was obtained from Sigma-Aldrich. Water was purified with a Milli-Q purification system (Millipore, USA).

2.2 Extraction of melatonin from rice grains

Melatonin extraction was performed on a basis of microwave-assisted extraction (MAE) technique in a Milestone Ethos 1600 (Sorisole, Italy) equipped with the vessels which are made of tetrafluoromethoxyl and lined with Teflon liners. The extraction of melatonin from rice grains was conducted according to the established procedure [17].

2.3 HPLC conditions

HPLC analyses were carried out on an Alliance HPLC 2695 system with a fluorescence detector (Waters 474 fluorescence detector), controlled by an Empower Pro 2002 data station (Waters, Milford, MA, USA). Separations were performed in

a reverse phase RP 18 Lichrospher Column (LiChroCART 250×4 mm (5 µm) from Merck) at 25 °C. The mobile phase was a binary solvent system consisting of phase A (2% acetic acid and 5% methanol in water) and phase B (2% acetic acid and 88% methanol in water) with a flow rate of 0.5 mL min^{-1}. The 25 min programmed gradient was as follows (%B): 0–5 min, 0–35%; 5–12 min, 35–40%; 12–15 min, 40%; 20–25 min, 45–50%. After each analysis, the column was washed with 100% B for 5 min and equilibrated with 0% B for 5 min. The established conditions for the fluorescence detector were as follows: excitation wavelength, 290 nm; emission wavelength, 330 nm; gain, 1000; attenuation, 16; injection volume, 10 µL.

2.4 UPLC analysis

UPLC analyses were carried out on an ACQUITY UPLC® H-Class system coupled to an ACQUITY UPLC® Fluorescence Detector (FD) and controlled by Empower™ 3 Chromatography Data Software (Waters Corporation). The excitation wavelength was set at 290 nm and the emission wavelength was set at 330 nm for the 2D scan. The FD sensitivity for the 2D scan was set at PMT gain 1, the data rate at 40 pts s^{-1} and the time constant at 0.1 s. Separations were performed at a temperature of 47 °C on a reverse phase RP 18 Acquity UPLC® BEH Column (Acquity UPLC® BEH 100×2.1 mm (1.7µm) from Waters Corporation).

The mobile phase was a binary solvent system consisting of phase A (water with 2% acetic acid) and phase B (acetonitrile with 2% acetic acid). The flow rate was 0.7 mL min^{-1}. The 4.0 minute gradient was as follows (%B): 0–1 min, 0%; 1–1.1 min, 0–10%; 1.1–2 min, 10%; 2–3 min, 10–20%; 3–3.5 min, 20–60%; 3.5–4 min, 60–100%. The column was subsequently washed with 100% B for 3 min and equilibrated with 0% B for 3 min. The injection volume was set at 3.0 µL.

2.5 Performance of the method

The analytical procedure of the chromatographic method for melatonin determination was carried out according to the recommendations of ICH Guideline Q2 (R1) [18] and suggestions in ISO 17025 [19]. Linearity, range, precision, detection and quantification limits of the method were established. The stability test inside the auto-sampler up to 4h for melatonin in standard solution (750 µg L^{-1}) was investigated. The peak signal was determined every 30 minutes and compared with those obtained from freshly prepared melatonin solution.

3. Results and discussion

In this study, UPLC method was developed and compared to the established HPLC method for the determination of melatonin in rice grain extracts. Different injection volumes, flow-rates and column temperatures were evaluated during the

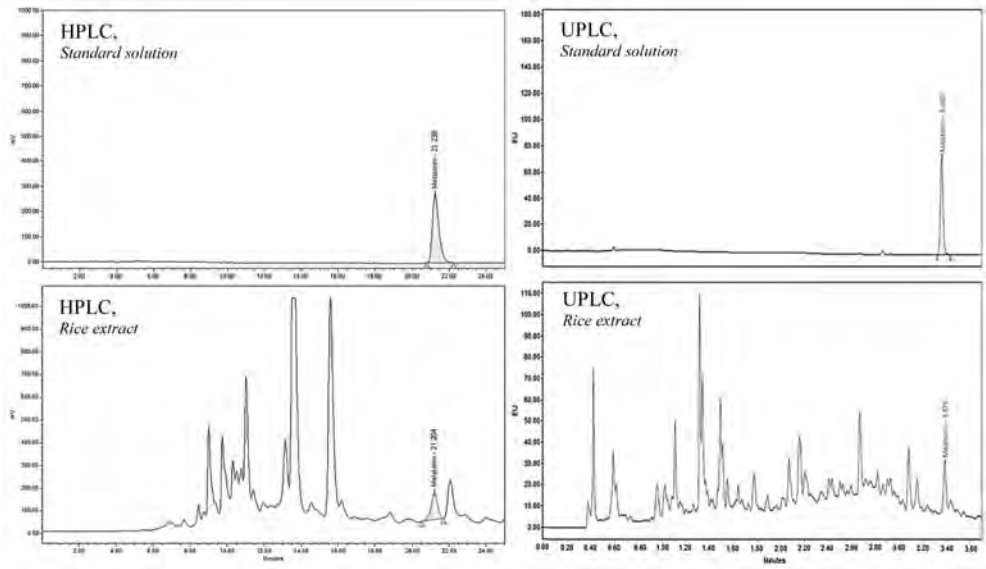


Fig. 2. Chromatograms of melatonin obtained by HPLC (left) and UPLC (right) analysis in standard solution (A) and in rice extract (B).

development of the UPLC method. To reach equivalent system performance compared with conventional HPLC, UPLC instrument manufacturer recommends using a low injection volume and 3.0 μL was preferred for the analysis. The retention time of melatonin decreased with the increase of column temperature as well as the peak resolution. Hence, the column temperature of 47 °C was chosen based on the maximum column temperature previously developed by the research group. At this column temperature, a rapid separation was achieved in less than 4 min using the flow rate of 0.7 mL min^{-1}. Fig. 2 shows the identification of melatonin in a standard solution (A) and rice grain extract (B) at the retention time of 21.2 and 3.4 min by HPLC and UPLC respectively. Apparently, the analysis run time reduced by two-thirds applying higher-pressure technology, which saved the eluents consumption up to 60%.

The validation properties for both chromatographic methods are listed in Table 1. The linearity of the calibration curves was validated by the high value of coefficient of determination of the regression analysis (R^2>0.99) for both chromatographic methods. The standard deviation and slope obtained from the regression analysis were used to calculate the limit of detection (*LOD*) and limit of quantification (LOQ). Since both limits were lower in UPLC than those in HPLC it is considered that the UPLC method is able to perform analysis in lower concentration of melatonin.

The precisions of the methods were evaluated by assessing repeatability (intra--day) and intermediate precision (extra-day). The precisions, expressed as coefficient of variantion (CV), of retention time were prominently better for UPLC

Table 1
Method validation parameters for determination of melatonin in rice grain extracts.

Parameter	HPLC	UPLC
Linear range concentration [μg L^{-1}]	0.5–15	0.5–20
Coefficient of determination R^2	0.996	0.999
LOD [μg L^{-1}]	1.15	0.73
LOQ [μg L^{-1}]	3.84	2.19
Precisions-retention time		
Repeatability (n = 9), CV [%]	0.99	0.28
Intermediate precisions (n = 3×3), CV [%]	1.23	0.12
Precisions-signal of height		
Repeatability (n = 9), CV [%]	0.93	0.97
Intermediate precisions (n = 3×3), CV [%]	2.05	1.40

analysis. Conversely, the peak-height repeatability were similar for both methods while the intermediate precision was slightly better for UPLC method. Hence, the analysis using UPLC is considerably more precise than using conventional HPLC method.

The stability of melatonin in standard solution of 50% methanol/water (v/v) was analysed by UPLC-FD during 4 h inside the auto-sampler. CVs of the peak area were roughly 15% applying regular temperature of auto-sampler (15 °C). Therefore, further studies evaluating lower temperature (4 °C), the use of antioxidants (50 ppm SO_2 and 5 ppm gallic acid) and direct injection of the solution from –20 °C storage location were performed. Fig. 3 shows the use of gallic acid could help to maintain the stability of melatonin during the time in auto-sampler waiting for the injection.

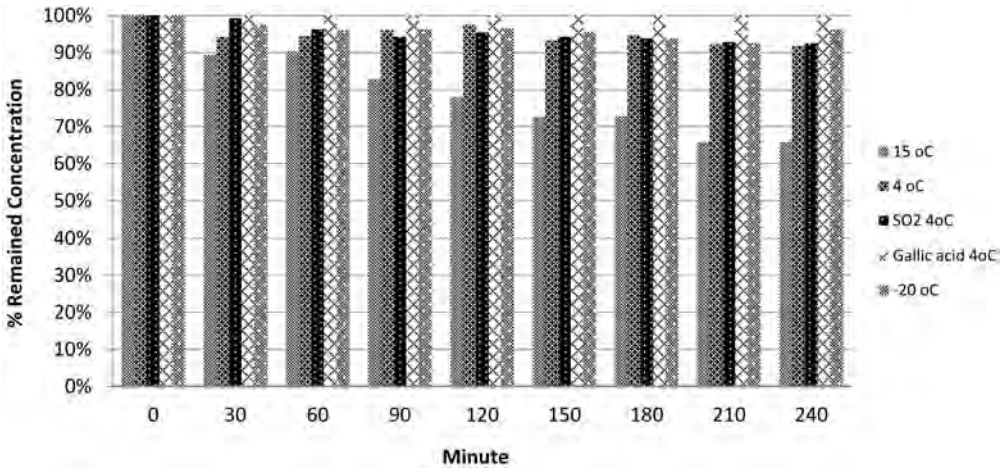


Fig. 3. Stability study of melatonin solution in auto-sampler.

4. Conclusions

Although several methods have been developed for determination of melatonin, both HPLC and UPLC proposed in this study can be recommended as rapid, precise and reliable methods for the determination of melatonin in rice grain extracts. However, UPLC can offer significant enhancements in speed and precisions compared with conventional HPLC. This technique is also more eco-friendly due to the lower consumption of eluent.

Acknowledgments

W.S. is grateful to the CIMB Foundation for a Ph.D. studentship through the CIMB Regional Scholarships 2012.

References

[1] Waters Corporation: *Ultra Performance LC™ by design.* Waters Corporation USA, 720000880EN:LL. &LW-UL 2004.
[2] Nováková L., Matysová L., Solich P.: *Talanta* **68** (2006), 908–918.
[3] Srivastava B., Sharma B.K., Baghel U.S., Yashwant, Sethi N.: *Int. J. of Pharmaceutical Quality Assurance* **2** (2010), 19–25.
[4] Kaufmann A., Butcher P., Maden K., Widmer M.: *Anal. Chim. Acta* **352** (2006), 13–21.
[5] Wang X.: *CNS Neurosci Ther.* **15** (2009), 345–357.
[6] Jung B., Ahmad N.: *Cancer Res.* **66** (2006), 978–993.
[7] Burkhardt S., Tan D.X., Manchester L.C., Hardeland R., Reiter R.J.: *J. Agric. Food Chem.* **49** (2001), 4898–4902.
[8] Reiter R.J., Manchester L.C., Tan D.X.: *Nutrition* **21** (2005), 920–924
[9] Talebianpoor M.S., Khodadoust S., Rozbehi A., Toori M.A., Zoladl M., Ghaedi M., Mohammadi R., Hosseinzadeha A.S.: *J. Chromatogr. B.* **960** (2014), 1–7.
[10] Ansari M., Rafiee Kh., Yasa N., Vardasbi S., Naimi S.M., Nowrouzi A.: *Daru* **18** (2010), 173–178.
[11] Mohammadi A., Rezanour N., Dogaheh M.A., Bidkorbeh F.G., Hashem M., Walker R.B.: *J. Chromatogr. B.* **846** (2007), 215–221.
[12] Kocadağlı T., Yılmaz C., Gökmen V.: *Food Chemistry* **153** (2014), 151–156.
[13] Pape C., Luening K.: *J. Pineal Res.* **41** (2006), 157–165.
[14] De la Puerta C., Carrascosa-Salmoral M.P., Garcia-Luna P.P., Lardone P.J., Herrera J.L., Fernandez--Montesinos R., Guerrero J.M., Pozo D.: *Food Chemistry* **104** (2007), 609–612.
[15] Iriti M., Rossoni M., Faoro F.: *J. Sci. Food Agric.* **86** (2006), 1432–1438.
[16] Van Tassel D.L., Roberts N., Lewy A., O'Neill S.D.: *J. Pineal Res.* **31** (2001), 8–15.
[17] Setyaningsih W., Palma M., Barroso C.G.: *J. Cereal Sci.* **56** (2012), 340–346.
[18] ICH: ICH Topic Q 2 (R1) *Validation of Analytical Procedures. Text and Methodology EMEA*, London, 2006.
[19] ISO/IEC 17025:2005 *General Requirements for the Competence of Testing and Calibration Laboratories.*

Synthesis and characterization of peptide stationary phases for liquid chromatography

Magdalena Skoczylas*, Szymon Bocian, Bogusław Buszewski

Department of Environmental Chemistry and Bioanalytics, Faculty of Chemistry, Nicolaus Copernicus University, Gagarina 7 St., 87 100 Torun, Poland, ✉ magdalena_skoczylas@hotmail.com

Keywords
amino acids
high performance liquid chromatography
HILIC
peptide stationary phases

Abstract
Bonded stationary phases are the predominant group of adsorbents commonly used in liquid chromatography. The diversity of naturally occurring compounds enforces to do research to find new generation of stationary phases. One of the interesting materials characterized by specific properties and the multiplicity of combinations of the structures are peptide stationary phases. The aim of the research was to develop methodology of peptide stationary phases synthesis, straightforward on the chemically modified silica surface, based on the Merrifield method. Two materials were synthesized and characterized: Amino-Gly and Amino-Ala, that contain immobilized dipeptide of glycine and alanine, respectively. The work presents also the application of received materials in HILIC mode for analysis of biologically active compounds of differently physicochemical properties.

1. Introduction

Peptides are one of the most important groups of compounds, which are necessary for the normal growing and functioning of every organism. The structure of the peptide is formed by a linear sequence of amino acids linked by peptide bonds. The number, structure and sequences of amino acids determine the biological activity of the polypeptide molecule [1].

Specific properties of different types of peptides cause widespread interest and applications in many fields of science, e.g. endocrinology [2], cosmetology [3], pharmaceutical and medical industry [4, 5]. Peptides due to their unique structural and biological properties exhibit also analytical application in liquid chromatography. The initiators of the chemical binding of the peptides on the surface of silica gel were Grushka and Scott [6, 7]. Commonly, peptide stationary phases are used in the analysis of both amino acids and dipeptides, as well as their isomers and derivatives. Amphoteric structure of amino acids makes it possible to use this type of materials as the zwitterionic stationary phases [8]. Peptide adsorbents significantly increase the collection of materials with a high orthogonality in two-dimensional mode RP/RP LC [9].

The application of peptide stationary phases currently involves a complex procedure for the synthesis of such materials. An interesting alternative synthesis

of peptide stationary phases is to use fundamental principles of Merrifield's method [10]. Ordered bonding of corresponding amino acids to chemically modified silica with aminopropyl groups can design a sequence of amino acids, and consequently the structure and properties of synthesized stationary phases. The diversity of amino acids which are forming a chemically bonded peptide chain, offers the possibility of obtaining adsorbents useful in any liquid chromatographic system.

2. Experimental

2.1 Reagents and chemicals

The solid support of laboratory-prepared stationary phases was Kromasil®100, with pore diameter 100 Å, pore volume 0.9 ml/g, surface area 310 m^2/g (Kromasil, Eka Chemicals, Sweden). Silica gel was chemically modified with aminopropyl groups with a coverage density 3 μmol/m^2. The following reagents were used for chemical modification of the silica support: Fmoc-Gly-OH, Fmoc-Ala-OH, *N,N`*-dicyclohexylcarbodiimide (DCCI), piperidine, dichloromethane (DCM), dimethylformamide (DMF) (Sigma-Aldrich), methanol, and toluene (J.T. Baker, Netherlands). Binary mobile phase consisting of acetonitrile, and water were applied in chromatographic analysis. Organic solvent were high purity for HPLC (Sigma--Aldrich). Water was purified using a Milli-Q system (Millipore, USA).

2.2 Instrumentation

The phases under study were packed into 125×4.6 mm stainless steel columns (Sigma Aldrich). Both columns were packed using Haskel INC (type DSF-122, USA) packing pump, under a pressure of 40 MPa. A liquid chromatograph was a Shimadzu Prominence (Japan) equipped with an autosampler (SIL-20A), column thermostat (CTO-10AS), a diode-array UV detector (SPD-M20A). Instrument control, data acquisition and processing were performed with LabSolution software for HPLC.

2.2 Synthesis of peptide stationary phases

The first step of synthesis was to remove the physically adsorbed water by 12-hour drying the silica gel modified with γ-aminopropyltrimethoxysilane at a temperature of 80±5 °C under reduced pressure. Next, prepared support was bonded with the first C-terminal amino acid. For this purpose, 5.6 g of derivatized silica gel and 40 ml of the suspension of 6 g of Fmoc-Ala-OH and 3g of DCCI (activator of carboxyl group) in DMF and DCM were placed in a glass flask heated to a temperature of 40 °C using an oil bath. The reaction was carried out during 24 h. The intermediate product of synthesis was rinsing with toluene and methanol on a Schott funnel, in order to eliminate the excess of reactants.

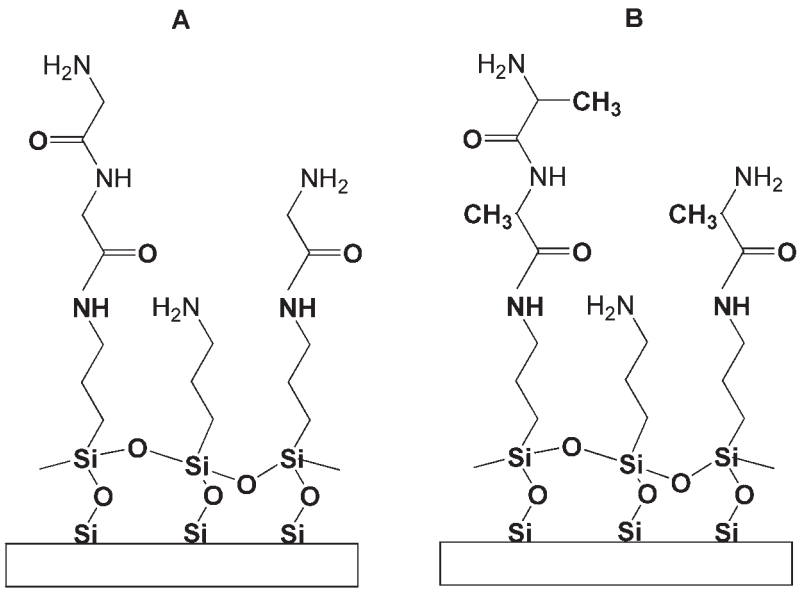


Fig. 1. Theoretical structures of the synthesized stationary phases: (A) Amino-Gly, (B) Amino-Ala.

The next stage of synthesis was the deprotection of the amino groups of the bonded amino acids. The removal of the Fmoc groups was carried out using 10 ml of 20% solution of piperidine and 40 ml of DMF. The resulting suspension was stirred for 15 min, and then the support was rinsing with toluene and methanol. The prepared adsorbent was dried in vacuum at 60 °C. The stationary phase comprising a first layer of alanine was subjected to further derivatization in the same manner analogous to that described above. Applied methods allowed to receive two stationary phases containing on their surface chemically bonded dipeptide of alanine (Amino-Ala) and glycine (Amino-Gly). The theoretical structures of the received stationary phases are shown in Fig. 1.

3. Results and discussion

Hydrophilic interaction liquid chromatography (HILIC) is a method designed strictly for the analysis of highly polar compounds. Hence, it is necessary to use stationary phases distinguished by hydrophilic properties. Peptide stationary phases significantly extend the group of materials which may be use in the HILIC mode. Confirmation of the suitability of the tested materials in a HILIC was done by the separation of the mixture of nucleosides, containing eight purine compounds and four pyrimidine analogs (Fig. 2).

The results obtained for each of the tested columns are diverse in terms of selectivity and efficiency. In the case of Amino-Gly, the separation was almost complete, in contrast to the Amino-Ala, for which chromatogram shows a partial

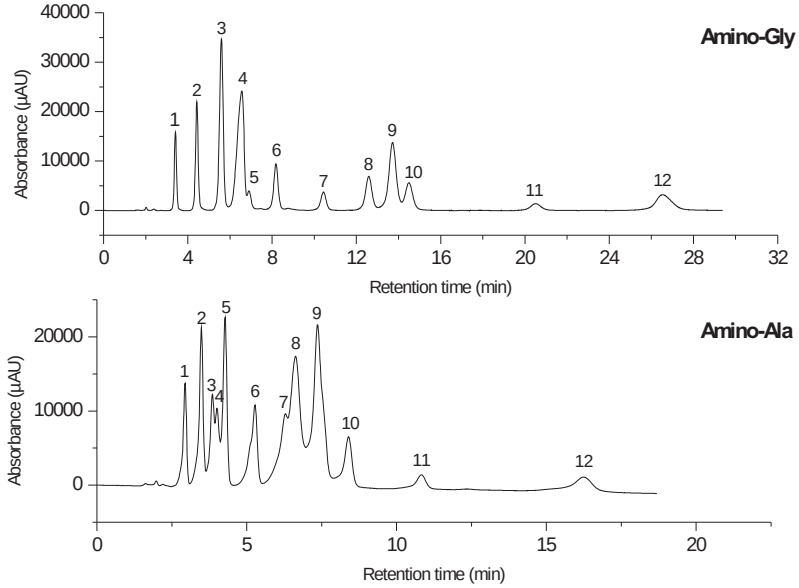


Fig. 2. The separation of nucleosides on Amino-Gly and Amino-Ala stationary phases: (1) 2-deoxythymidyne, (2) 1-methyladenosine,(3) 1-methylinosine, (4) adenosine, (5) uridine, (6) 7-methylguanosine, (7) 1-methylguanosine, (8) pseudouridine, (9) cytidine, (10) 8-bromoguanosine, (11) N^2-methylguanosine, (12) guanosine. Conditions: mobile phase: ACN/H_2O (92/8); flow rate: 1 mL min^{-1}; 30 °C, λ = 254 nm.

separation. As expected, the reason of the observed results is an additional methyl group in the alanine molecule, which gives a slight hydrophobic property of surface of the stationary phase. Therefore, hydrophilic analytes are less retained on the stationary phase with chemically bonded dipeptide of alanine, which is reflected in reduced retention times of the individual compounds. The retention mechanism based on the electrostatic interactions, hydrogen bonding, dipol-dipol and weak hydrophobic interactions. The efficiency of the tested columns reaches 6012 and 8530 theoretical plates, for Amino-Gly, and Amino-Ala, respectively, at the column length of 125 mm.

4. Conclusions

The application of the basic principles of the Merrifield method enabled to elaborate peptide synthesis methodology directly onto the modified silica gel surface. Two synthesized stationary phases contain dipeptide of glycine and alanine. Peptide stationary phases were successfully applied in HILIC system for the analysis of polar compounds. The nucleosides characterized by respective retention times and almost complete separation. Despite the slight difference in

the structure (methyl group) of the two stationary phases differences were observed in the retention of the individual compounds. In conclusion, the designing structures of the peptide adsorbents on selected chemical properties, depending on the application needs, allow to obtain highly selective stationary phase for liquid chromatography

Acknowledgments

This study was supported by grants from Ministry of Science and Higher Education Iuventus Plus IP2014 003673.

References

[1] Murray R.K., Granner D.K., Mayes P.A., Rodwell V.W.: *Biochemia Harpera*. Warszawa, Wydawnictwo Lekarskie 1994, s. 35–69, 415–418.
[2] Kączkowski J.: *Podstawy Biochemii*. Warszawa, Wydawnictwo Naukowo-Techniczne 1999, s. 48–78.
[3] http://www.pkik24.pl/kosmetologia-i-kosmetyka/peptydy-biomimetyczne-i-czynniki-wzrostu-w-kosmetologii-i-medycynie-estetycznej (accessed 12th June, 2015)
[4] Smolarczyk R., Cichoń T., Szala S.: *Postępy Higieny i Medycyny Doświadczalnej* **63** (2009), 360–368.
[5] Cui Y., Kim S.N., Naik R.R., Mcalpine M.C.: *Accounts of Chem. Res.* **4**5 (2012)**,** 696–704.
[6] Fong G.W-K., Grushka E.: *Anal. Chem.* **50** (1978), 1154–1161.
[7] Kikta E.J., Grushka E.: *J. Chromatogr.* **135** (1977), 367–376.
[8] Schen A., Guo Z., Yu L., Cao L., Liang X.: *Chem. Commun.* **47** (2011), 4550–4552.
[9] Xue M., Huang H., Ke Y., Chu Ch., Yu jin, Liang X.: *J. Chromatogr.* **1216** (2009), 8623–8629.
[10] McMurry J.: *Chemia organiczna*. Warszawa, Wydawnictwo Naukowe PWN 2000, s. 1060–1098.

Application of electronic nose based on fast GC for comparison of aroma profiles of homemade cherry "Nalewka" made with addition of different amount of sugar

Magdalena Śliwińska*, Paulina Wiśniewska, Tomasz Dymerski, Waldemar Wardencki, Jacek Namieśnik

Department of Analytical Chemistry, The Chemical Faculty, Gdansk University of Technology, 11/12 Narutowicza St., 80-233 Gdańsk, Poland ✉ *m.e.sliwinska@gmail.com*

Keywords
addition of sugar
aroma profile
electronic nose
fast GC
nalewka

Abstract
"Nalewka" is a traditional Polish homemade spirit beverage and there are many different recepies for the production of nalewkas. However, it is not known what amount of sugar can have influence of their aroma profiles. There is little information on the comparison of aroma profiles of Polish cherry nalewkas made with addition of different amount of sugar. For this purpose electronic nose based on fast GC has been used. Electronic nose was equipped with two parallel connected columns with different stationary phases (non polar and medium polar) coupled with two ultrasensitive flame ionization detectors (μFID). Three data analysis were used, namely Principal Component Analysis (PCA), Discriminant Function Analysis (DFA) and Soft Independent Modeling of Class Analogies (SIMCA). Application of electronic nose based on fast GC allows compare off aroma profiles of all cherry nalewkas made with addition of different amount of sugar (10 g, 25 g and 50 g). The results obtained by the use of PCA analysis and SIMCA classification are consistent with all the data obtained by means of interpretation of the chromatograms.

1. Introduction

"Nalewka" is a traditional Polish homemade spirit beverage. Their production is Polish centuries-long tradition. For the preparation of nalewka three main ingredients are used: fruits (sometimes herbs or roots), sugar and spirit. There are many different recepies for the production of nalewkas. However the most common manufacturing process consist of following three steps. Maceration is a process in which ingredients are covered with alcohol to exude their flavor, aroma, and color [1]. In the first stage fruits are covered with sugar in the jars. After few days the contents of the jar is flooded with pure spirit. This maceration process most often takes 3–8 weeks. The last step is filtered the contents of jar and the remaining liquids poured into the bottles. The alcoholic strength of the final

product is usually 40% alcohol by volume [2, 3]. Traditional homemade nalewkas do not contain artificial flavors or artificial colors.

So far, nalewkas have been mainly studied by the use of EPR, NMR, and UV-VIS spectroscopy [1]. In the literature there is no information concerning the examination of these drinks and similar beverages (liqueurs) using electronic nose device [4].

Electronic nose technology is used for many years in alcohol investigations. So far, electronic noses based on different sensors: conductometric, amperometric, colorimetric and piezoelectric have been commonly used [5–9]. Electronic nose is mainly applied in the spirits industry for the quality control, authenticity assessment and process monitoring [9–11]. Recently some information appears about the utilization of electronic nose based on fast GC. In this specific type of equipment the data obtained from the selection of chromatographic peak areas with the highest discriminate power is used as an input data for further chemometric analysis. The chromatograms consist the characteristic aroma profiles called fingerprints. They can be treated as a responses of the device for an interaction with a gaseous mixture introduced into the chromatographic system [5, 12]. So far, there are few information about utilization of this device in studies of alcoholic beverages. In literature informations can be found about application of this type of electronic nose in comparison of aroma profiles of whiskey originating from different distilleries [13] and also grape wine age and brand [14].

Regarding the fact that there are no scientific reports about the comparison of aroma profiles of Polish cherry nalewkas in terms of different amount of sugar it is highly important to enrich the status of the knowledge in this scientific discipline. The main aim of this paper was to conduct the studies related to the application of electronic nose based on fast GC for comparison of aroma profiles of homemade cherry nalewkas made with addition of different amount of sugar. For this purpose three statistical methods of data analysis, i.e. PCA, DFA, and SIMCA and comparison of all the data obtained by means of interpretation of the chromatograms were used.

2. Experimental

2.1 Samples and sample preparation

Homemade nalewkas have been prepared according to the following scheme (Fig. 1). Fruits (50 g) was placed in three jars . Then the fruits in jars were covered with sugar (50 g / 25 g / 10 g). Subsequently the jars were sealed tightly for 3 days. The next step was the addition of 25 ml of spirit and 25 ml of deionized water to each jar. Again, jars were sealed and left in a dark place for a period of four weeks. After this time, fruits were filtered through gauzes, then the remaining liquids poured into the bottles. Cherries (*Prunus cerasus*) used for the production of nalewkas were harvested in August 2014 in Pomeranian Voivodeship, Poland.

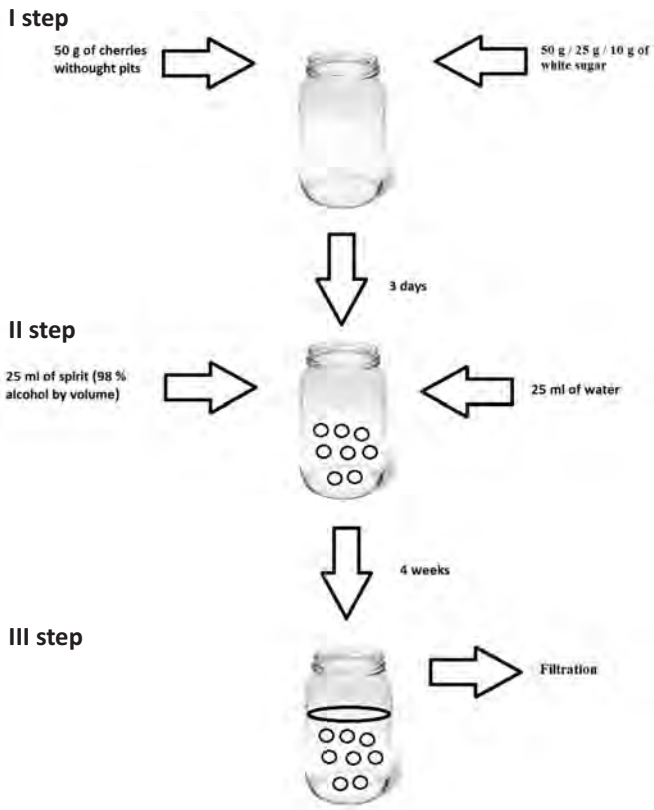


Fig. 1. Scheme of the procedure of nalewka preparation.

Fruits have been thoroughly washed and were used to produce nalewkas after harvest. For the production of nalewkas spirit (Spirytus Gdański, Destylarnia Sobieski, Starogard Gdański, Poland) and sugar (Cukier Biały, Diamant, Poznan, Poland) have been also used.

Samples were prepared in the proportion of 1 ml of nalewkas and 4 ml of deionized water and transferred into 20 ml vials. All samples were sealed with caps closing with 20 mm thick PTFE/silicone membrane.

2.2 Chemicals

Deionized water of high purity from MilliQ A10 Gradient/Elix System (Millipore, USA) was used throughout the research.

2.3 Instrumentation

The electronic nose based on fast GC (Heracles II, Alpha M.O.S., France) equipped with two parallel connected columns with different stationary phases coupled to

two ultra sensitive flame ionization detectors (μ-FIDs) was used for the analysis. The first nonpolar column was MTX-5 (10 m × 0.18 mm × 0.4 μm film thickness) and the second column with medium polar stationary phase MXT-1701 (10 m × 0.18 mm × 0.4 μm film thickness) was applied. The vial with the liquid sample was introduced into the autosampler (Odour Scanner HS 100; Gerstel, Germany). Gas gained from the headspace of sample was used for the analysis. The incubation was conducted during the time of 20 min and in temperature of 40°C. Agitation speed was maintained on the level of 500 rpm. After incubation process the gas sample was taken from headspace of sample and transferred from vial to the GC injector port kept at 200 °C. The separation of analytes was performed using the following chromatographic temperature program: initial temperature 70 °C (kept for 18 s) then ramped at 2 °C/s to 270°C (kept for 30 s). The hydrogen N5.0 (Linde Gaz, Poland) class was used as a carrier gas. Detector temperature was 270 °C in. During the research for each sample 3 repetitions were performed.

2.4 Data analysis

Three chemometric methods, Principal Component Analysis (PCA), Discriminant Function Analysis (DFA) and Soft Independent Modeling of Class Analogies (SIMCA) have been used for data analysis. All these methods were performed using Alpha Soft (V12.4, Alpha M.O.S, France).

3. Results and discussion

Principal Component Analysis is a chemometric linear unsupervised and pattern recognition technique used for analyzing and reducing the dimensionality of numerical datasets in a multivariate problem [15, 16]. The PCA method was performed to identify patterns of correlation with individual composition variables involved in the discrimination among three groups of cherry nalewkas made with addition of different amount of sugar (10 g, 25 g or 50 g). In Fig. 2 the PCA score plot of comparison aroma profiles of different cherry nalewkas made with different sugar concentration is represented. The PCA result shows the first two principal components accounted for 99.91% of total variance. A clear discrimination of nalewkas made with the addition of 10 g of sugar and other nalewkas based on their volatile composition can also be indicated. The points on PCA plane which are the representation of nalewkas samples appears in two group classes: these which are representing nalewka made with 10 g of sugar are situated on the left side of the diagram whereas the points connected with samples of nalewkas made with the addition of 25 g and 50 g of sugar are located on the right part of the graph.

The DFA (Discriminant Function Analysis) is another method for grouping data and differentiating between groups. The DFA method is used when the assumptions of a linear regression are met. The cases are classified into groups using

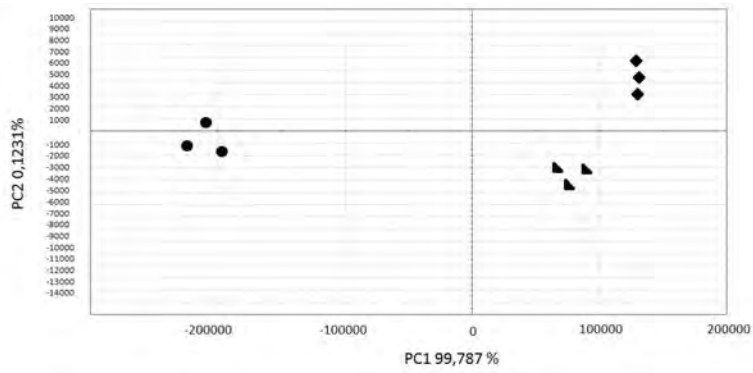


Fig. 2. The PCA plots of comparison aroma profiles of three groups of cherry nalewkas (● 10 g, ◣ 25 g, ◆50 g) using electronic nose based on fast GC.

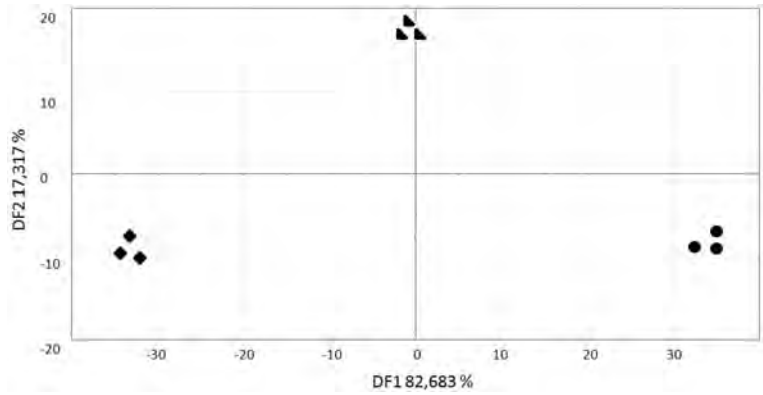


Fig. 3. The DFA plots of comparison aroma profiles of three groups of cherry nalewkas (● 10 g, ◣ 25 g, ◆ 50 g) using electronic nose based on fast GC.

a discriminant prediction equation to examine differences between or among groups. This statistic method determines whether groups differ from each other and identifies the possible subgroups [9]. Using DFA method it was possible to identify DF1 and DF2 which explain respectively 99.787% and 0.123% of the variability of the initial data set. In contrast to PCA result, DFA data is divided into three parts. Points belonging to nalewka made with the addition of 10 g of sugar are situated on the left side of the graph, on the middle are points belonging to nalewka made with the addition of 25 g sugar and on the right side of graph are points belonging to group of nalewka made with the highest amount of sugar. Application of DFA analysis allows for discrimination between all three groups of nalewkas (Fig. 3) Due to the both PCA and DFA results it was necessary to check other available chemometric methods.

The SIMCA (Soft Independent Modeling of Class Analogy) classification and the PCA analysis can be used to visualize the distance between the classes [17, 18].

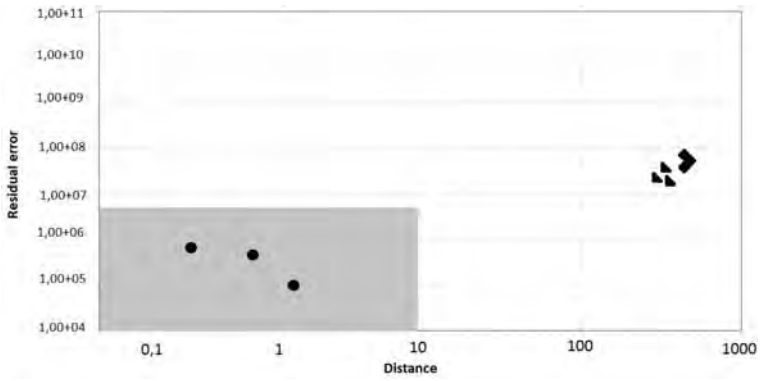


Fig. 4. The SIMCA classification plot of three groups of cherry nalewkas (● 10 g, ◣ 25 g, ◆ 50 g) using electronic nose based on fast GC. Reference group: cherry nalewkas made with addition of 10 g of sugar.

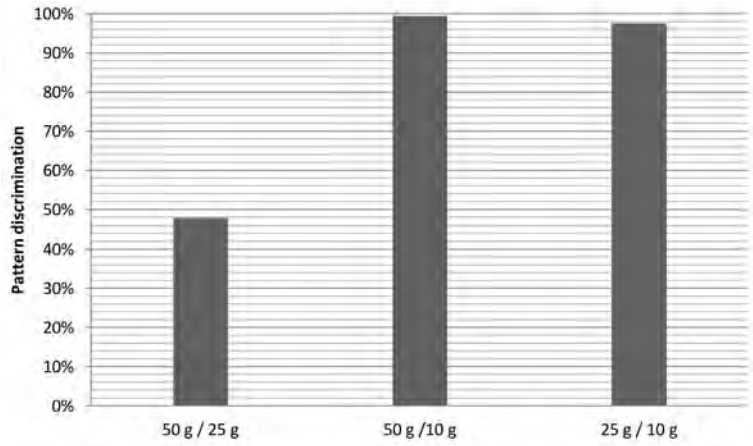


Fig. 5. The result of comparison pattern discrimination index each of three groups to cherry nalewkas.

The SIMCA method is used to compare unknown samples with the reference group. In SIMCA model such comparisons are made with one specific group form database. The SIMCA classification plot corresponding to the same input data applied to the PCA and DFA methods is shown in Fig. 4. The area corresponding to the given class is shown on each plot as gray rectangle in the left corner. As shown on the plot, all points belonging to nalewka made with the addition of 10 g of sugar were assigned correctly to the corresponding class. In Fig. 4 validation score is 90 and it was possible to obtain a significant distinction between three groups of nalewkas. Very small distance between the two groups of nalewkas samples made with the addition of more amount (25 g and 50 g) sugar can also be observed. The result of SIMCA classification is similar to the results obtained using PCA method.

The verification on chemometric methods regarding suitability were performed by the use of comparison of aroma profiles of all the data obtained in chromatograms interpretation (Fig. 5). Pattern discrimination index is only 47.78% in comparison between nalewkas samples made with the addition of 25 g and 50 g of sugar. The highest values of pattern discrimination were achieved between comparison of nalewka samples made with the addition 10 g to 50 g and 10 g to 25 g of sugar. For this reason, it can be concluded that a large amount of added sugar does not affect the differences in the aroma profiles of nalewkas. The results obtained using PCA analysis and SIMCA classification are consistent with all the data obtained by means of interpretation of the chromatograms (comparison aroma profiles of nalewkas).

4. Conclusion

Application of the electronic nose based of fast GC allows for the comparison of aroma profiles of homemade cherry nalewkas made with different sugar content. Using three different chemometric methods and the comparison of chromatographic results it was possible to verify these methods which were consistent with the chromatographic results. The PCA and SIMCA analysis demonstrated that the profiles of aroma profile of cherry nalewkas made with the least amount of sugar were significantly different from the others nalewkas made with addition of 25 g and 50 g of sugar. The DFA analysis showed that it is possible to distinguish the aromatic profiles of all examined nalewkas. However it is not possible to find the similarities in the aroma profiles in nalewkas made with addition of 25 g and 50 g of sugar. In addition, it can be concluded that a large amount of added sugar does not affect the differences in the aroma profiles of nalewkas. The use of electronic nose based on fast GC can be useful in the production of nalewkas, liqueurs and other spirits beverages in terms of the quality analysis and authenticity assessment.

Acknowledgments

The authors acknowledge the financial support for this study by the Grant No. 2012/05/B/ST4/-01984 from National Science Centre of Poland.

References

[1] Polak J., Bartoszek M.: *J. Food Comp. Anal.* **40** (2015), 114–119.
[2] Fiedoruk A.: *Polskie nalewki i wódki.* Bielsko Biała, Dragon 2006.
[3] Kowalski J.: *Wielka księga nalewek.* Warszawa, Olesiejuk 2011.
[4] Śliwińska M., Wiśniewska P, Dymerski T., Wardencki W., Namieśnik J.: *Flav. Fragr. J.* **30** (2015), 197–480.
[5] Winquist F., Holmin S., Krantz-Rückler C., Wide P., Lündström I.: *Anal. Chim. Acta* **406** (2000), 147–157.
[6] Stetter J. R., Findlay M. W., Schroeder K. M., Jr. Yue C., Penrose W. R.: *Anal. Chim. Acta* **284** (1993), 1–11.

[7] Saevels S., Lammertyn J., Berna A. Z., Veraverbeke E. A., Di Natale C., Nicolaï B. M.: *Postharvest Biol. Technol.* **30** (2003), 3–14.
[8] Schaller E., Bosset J. O., Escher F.: *LWT-Food Sci. Technol.* **31** (1998), 305–316.
[9] Śliwińska M., Wiśniewska P, Dymerski T., Namieśnik J., Wardencki W.: *J. Agric. Food Chem.* **19** (2014), 1423–1448.
[10] Kojima H., Araki S., Kaneda H.J.: *J. Am. Soc. Brew. Chem.* **63** (2005), 151–157.
[11] Lozano J., Arroyo T., Santos J.P., Cabellos J.M., Horrillo M.C.: *Sens. Actuators B* **133** (2008), 180–186.
[12] Dymerski T., Chmiel T., Wardencki W.: *Rev. Sci. Instrum.* **82** (2011), 1–32.
[13] Kundu K., Mitra S., Mazumdar D., Pal S.K.: *Perception and Machine Intelligence.* New York Springer 2012.
[14] Yang Y., Zhao Y., Zhang S., Ni Y., Zhan J.: Qualitative analysis of age and brand of unblended brandy by electronic nose. In: *Computer and Computing Technologies in Agriculture. Vol. V.* Li D., Chen. Y. (edits.). Berlin, Springer 2012, p. 619–628.
[15] Ragazzo-Sanchez J.A., Chalier P., Chevalier-Lucia D., Calderon-Santoyo M., Ghommidh C.: *Sens. Actuat. B* **140** (2009), 29–34.
[16] Ghasemi-Varnamkhasti M., Rodríguez-Méndez M.L., Saeid Mohtasebi S., Apetrei C., Lozano J., Ahmadi H., Hadi Razavi S., de Saja J. S.: *Food Control* **25** (2012), 216–224.
[17] Olsson J., Börjesson T., Lundstedt T., Schnürer J.: *Int. J. Food Microbiol.* **10** (2000), 167–178.
[18] Ghasemi-Varnamkhasti M., Saeid Mohtasebi S., Rodríguez-Méndez M.L., Gomes A.A., Ugulino Araújo M.C., Galvão R.K.H.: *Talanta* **89** (2012), 286–291.

Non-traditional material for the development of electrochemical DNA biosensor in pharmaceutical analysis

Jana Svítková*, Silvia Švikruhová, Ľubomír Švorc

Slovak University of Technology in Bratislava, Faculty of Chemical and Food Technology, Institute of Analytical Chemistry, Radlinského 9, 812 37 Bratislava, Slovak Republic ✉ jana.svitkova@stuba.sk

Keywords
biosensor
boron-doped diamond electrode
DNA
drugs
interaction

Abstract
The active substances are subjected to numerous tests for detecting the levels of toxicity and the therapeutic levels. Another important aspect of the biological studies of pharmaceuticals as well as pharmaceutical development processes consists in the interaction of the drugs with DNA. For the investigation of interaction with DNA four different drugs (amlodipine, epinephrine, penicillin V, and paracetamol) and caffeine as representative alkaloids with the encouraging effect of the central nervous system were chosen. The aim of work was study and optimization of the experimental conditions for preparing a biosensor using the boron-doped diamond (BDD) electrode as a non-traditional electrochemical transducer. The effect of immobilization of DNA at the electrode surface was explored. Two ways of immobilization of DNA were compared (spontaneous adsorption and/or accumulation at the BDD electrode surface). On the basis of electrochemical measurements performed after incubation of the modified electrode in a drug solution, the mutual interaction can be observed. It probably leads to breaking of the DNA double helix, which is then washed away from the electrode.

1. Introduction

Biosensors are widely used in clinical, environmental and food analysis. The detection of damage to the DNA belongs to one of the specific fields of analysis with utilization of DNA-based biosensors [1]. DNA damage is always caused by many factors, either endogenous or exogenous origin, such as physical and chemical agents, drugs, radiation [2]. Investigation of interactions between drugs and DNA needs to devise a proper construction of biosensor, which will be able to serve as a reliable indicator of damage. This work focuses on the development and application of a novel and simple electrochemical DNA biosensor for rapid assessment of reactivity of surface-bound DNA. Boron-doped diamond (BDD) is a modern electrode material which opens new possibilities of electrochemical investigations due to its excellent features, such as the wide potential window in aqueous solutions, low background current, long-term stability of response, low

sensitivity to dissolved oxygen and a good resistance to surface fouling due to weak adsorption, mechanical robustness and compatibility with biological materials. BDD electrode is usually used as an electrochemical sensor with excellent sensitivity and selectivity [3].

As far as we know, there are not many works concerning the use of BDD electrochemical transducer for immobilization of DNA and subsequent examination of interactions with drugs.

2. Experimental

2.1 Reagents and chemicals

Various drug standards (purity from 98% to 99.5%) and dsDNA (fish sperm) were obtained from Sigma-Aldrich. The stock solution of DNA (1 mg/mL) was prepared by dissolving suitable amount of solid standard in 2 mL of sodium hydroxide (0.2 mol/L) followed by dilution with deionized water to the desired volume. The stock solutions of drugs (1×10^{-3} mol/L) were prepared by dissolution of respective amount of standard using deionized water and stored at 4 °C. Working solutions of lower concentrations were freshly made on the day of experiment by diluting the stock solution with supporting electrolyte. The supporting electrolyte was 0.1 mol/L phosphate buffer solution (PBS, pH = 6.9) which was made by usual way by dissolution of $Na_2HPO_4.12H_2O$, $NaH_2PO_4.10H_2O$ and KCl in deionized water. The stock mixture solution of 1×10^{-3} mol/L $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ (Lachema, CZ) was prepared by dissolution in deionized water and added to the required volume with PBS. Other chemicals were used of analytical reagent grade purity.

2.2 Instrumentation

All electrochemical experiments were conducted in a three electrode system. The cell consisted of Ag/AgCl (3 mol/L KCl) as the reference electrode, a platinum wire as the counter electrode and BDD electrode was prepared at the Institute of Electronics and Photonics, FEI STU (Bratislava) (parameters: CH_4/H_2 ratio = 1% and the B/C ratio = 10 000 ppm, deposition time = 2 h) served as the working electrode. Electrochemical measurements were carried out using an Autolab PGSTAT 302N (Metrohm Autolab) potentiostat/galvanostat controlled with the NOVA software of 1.10 version. The electrochemical properties of the unmodified and the modified BDD electrode were studied by CV and EIS in 1×10^{-3} mol/L $[Fe(CN)_6]^{3-/4-}$ in 0.1 mol/L PBS (pH = 6.9). All pH values were measured with pH meter Model 215 (Denver Instrument, USA), which was weekly calibrated with standard buffer solutions.

3. Results and discussion

BDD electrode was applied as non-traditional electrochemical transducer for the construction of DNA biosensor. The biosensor was used for investigation of the interaction of immobilized DNA at the BDD electrode surface with selected drugs at various incubation times (5–20 min). Based on the results of cyclic voltammetry at the DNA/BDD biosensor after incubation in particular drug the relative value of the current peak ΔI_{rel} [%] was calculated. The higher the ΔI_{rel}, the lower damage to the DNA, and thus less DNA was "taken down" from the electrode surface owing to the particular drug. The remaining portion of DNA (surv DNA) after the incubation of the biosensor in the drug was expressed as the normalized biosensor responses, which were calculated according to the equation:

$$\Delta I_{rel} = [(I_{surv\,DNA} - I_{bare\,BDDE})/(I_{DNA} - I_{bare\,BDDE})] \times 100 \quad (1)$$

the CV of anodic current observed at the electrode modified layer of DNA (before incubation I_{DNA}, and after incubation $I_{suvr\ DNA}$) for the potential, which is corresponding to the maximum peak current of 1×10^{-3} mol/L $[Fe(CN)_6]^{3-/4-}$ at the bare BDD electrode ($I_{bare\,BDDE}$).

Table 1 shows the percentage of damage to DNA at the BDD electrode surface with normalized values accomplished before and after incubation in the drug. With increasing incubation time, ΔI_{rel} values decrease and exhibit more damage to DNA.

EIS technique provided information about the resistance of the BDD electrode surface/biosensor, on the basis of which it was possible to characterize the changes in the DNA structure. To confirm damage to DNA with the drugs differential pulse voltammetry was chosen. After incubation the biosensor in a drug solution, with increasing incubation time the signal change of current response of guanine ($E_{p(G)}$ = 0.90 V) and adenine ($E_{p(A)}$ = 1.15 V) was evidenced. From these results we can conclude that the intercalation of a drug in DNA strands. The

Table 1
The ΔI_{rel} data obtained at unmodified and DNA – modified BDD electrode after incubation in solutions of drugs ($c = 1\times10^{-4}$ mol/L) at different times (5–20 min).

Incubation time [min]	ΔI_{rel}				
	Amlodipine	Epinephrine	Caffeine	Paracetamol	Penicillin V
5	44.4	35.1	41.2	40.7	42.9
10	31.6	22.4	34.8	19.4	35.0
15	24.4	25.4	28.0	13.4	32.3
20	14.4	5.4	10.6	9.9	21.7

intercalation can also be considered by a change in oxidation current response of guanine and decrease of charge transfer resistance R_{ct}.

4. Conclusions

All controlled substances showed certainly interaction with DNA. Development of the DNA biosensor represents a new approach for monitoring DNA damage due to effect of drug using BDD electrode. This study may serve as a useful tool for further experiments concerning the binding of biomolecules at the BDD electrode surface and the study of the interactions with the substances naturally present in the environment, which may demonstrate the negative effects on living organisms and for clinical and pharmaceutical analysis.

Acknowledgments

This work was supported by Scientific Grant Agency VEGA of the Slovak Republic (Projects No. 1/0051/13 and 1/0361/14).

References

[1] Labuda J., Vyskočil V.: DNA/electrode interface. Detection of damage to DNA using DNA-modified electrodes. In: *Encyclopedia of Applied Electrochemistry.* Ota K., Kreysa G., Savinell R. F. (edits.). Berlin, Springer 2014.
[2] Carlos S., Oliveira B., Oliveira-Brett A.M.: *Langmuir* **28** (2012), 4895–4901.
[3] Švorc Ľ., Kalcher K.: *Sens. Actuators B* **194** (2014), 332–342.

Toxicological assessment of food packages extracts

Błażej Kudłak, Jacek Namieśnik, Natalia Szczepańska*

Department of Analytical Chemistry, Faculty of Chemistry, Gdańsk University of Technology, 11/12 Narutowicza Str., 80-233 Gdańsk, Poland ✉ *natszcze@student.pg.gda.pl*

Keywords
chronic exposure
endocrine disrupting compounds
food packaging
migration
toxicity

Abstract
In the scientific literature related to the widely understood issue of packaging materials designed to have contact with food (Food Contact Materials) there is much information on raw materials used for their production, their physiochemical properties, types and parameters. There is also a great number of publications on the management and disposal of used packaging. Unfortunately, not much attention is given to the matters concerning migration of toxic substance from packaging and its actual influence on the body of the final consumer of the food packed, even though health protection and food safety are the priority tasks. The goal of tests was to estimate the impact of foodstuff packaging type, production and storage conditions on the degree of leaching of potentially toxic compounds to foodstuffs with the use of the acute toxicity test Microtox®.

1. Introduction

In the scientific literature, containing the latest research findings related to the monitoring of xenobiotics, as well as the one which addresses different toxicological aspects, one can find increasingly more information on the sources and the volume of emissions released by everyday objects. One of the priority issues in this area of research is, without a doubt, the one of migration of low molecular weight compounds from the packaging to the food stored inside. The multiannual research carried out in numerous research centres enabled, first and foremost, the identification of main groups of compounds leaching from the inner layer of the package as well as the conditions affecting the intensification of this process. Among the most common analytes, both in simulants and in food samples stored in cans or in the polymeric materials, the following types can be distinguished: Bisphenol A, Bisphenol A diglycidyl ether, its derivatives and the compounds of phthalates [1]. The main source of contamination is the special coatings used to protect the inner layer of the packaging from corroding and also to protect food from direct contact with the material used in producing the container. Nowadays, it is known that as a result of technological processes and its interaction with food ingredients, migrating compounds may be subject to a transformation into

Table 1
Normative figures for the "specific migration" (SML) parameter established for chosen chemical compounds.

Compound	SML [μg/kg foodstuffs or food simulants]
Bisphenol A	600
Sum of Bisphenol A diglycidyl ether and their hydrolysed derivatives	9000
Sum of Bisphenol A diglycidyl ether and their hydrochloric derivatives	1000

various types of derivatives of physicochemical and toxicological properties different then the initial [2].

The main criterion for approving packaging materials for contact with food are the toxic properties of the substances used to produce a given material and the degree of migration and specific migration determined on the basis of normative values of tolerable daily intake. Unfortunately, however, the standard guidelines for assessing the impact of packaging on food quality only take into account the numerical values of the parameter "global migration" and "specific migration limits" fixed for a small group of compounds. In the Table 1 results on normative limits for specific migration established by experts of the Scientific Committee for Food are presented [3].

Such approach raises many objections, given that the results of a recent study showed that apart from the monitored compounds leaching from the surface of the package, there are also other contaminants and their derivatives formed by interaction with food components and as a result of technological procedures applied. All unidentified contamination is described in the literature as NIAS (Non-Intentionally Addend Substances) [1]. The inability to accurately identify and quantify all the substances released into the food which then enter the body orally and the lack of adequate toxicological knowledge make it impossible to assess the real danger faced by consumers and predict the possible consequences associated with a long-term exposure. What makes the situation even worse is that among a large group of synthetic compounds used during the production of protective layers that can potentially enter the food, the vast majority exhibits properties similar to contaminants from the endocrine disrupting compounds group [2]. The presence of compounds affecting the proper functioning of the endocrine system in the environment poses many problems for researchers. For many years, many research centres have been conducting research to explain the processes and mechanisms by which these compounds modify the functioning of the living organism. Unfortunately, the knowledge about the complex mechanisms of toxic activity of these compounds is still very limited. What makes the situation even more serious is the fact that, according to numerous experimental data carried out both *in vitro* and *in vivo*, the dose-response for these

compounds is not monotonic and hence the usage of data obtained by exposing animal organisms to high doses of xenoestrogens in order to assess the risk of toxic effects in humans exposed to low doses over a longer period of time can be seriously flawed. Hence, the application of traditional principles and assumptions on which the assessment of the health risks for other contaminants is based cannot be used to evaluate the hormonally active compounds [4]. Moreover, during the toxicological assessment of hormone derivative compounds it is also necessary to take into account the interaction that can occur between these contaminants. According to numerous studies, the presence of several xenoestrogens of low or even very low concentration levels may result in a toxic effect. The combined action of biologically active compounds may reduce (antagonism) or reinforce (synergism) the observed toxic effect [5]. In view of such knowledge it is obvious that based on the composition of the sample alone it is not possible to estimate the risk posed by the presence of a mixture of xenobiotics occurring at different levels of content. Determining the total pollution level of the sample is only possible when one applies methods that utilize living organisms as active elements during the test. The goal of tests was to estimate the impact of foodstuff packaging type, production and storage conditions on the degree of leaching of potentially toxic compounds to foodstuffs with the use of the acute toxicity test Microtox®.

2. Experimental

A review of the data from the literature indicates to the fact that factors which have the biggest influence on the intensity of the process of migration of contamination from the packaging material is the temperature, the contact time and physiochemical properties of the medium prepared [2, 6]. Three stimulants of specific kinds of food have been chosen in relation to the methodologies used in order to assess global migration, as described in the EU Directives 82/711/EEC and 85/572 EEC: distilled water for aqueous foods with a pH above 4.5; acetic acid at 3% in distilled water for acidic aqueous food with pH below 4.5; ethanol at 5% for alcoholic food. Xenobiotics migration from epoxy resins was studies at 3 different temperatures: room temperature (RT) (t = 25 °C, 65 °C, and 121 °C) and specified food cans were doubly heated at 65 °C and 121 °C after 5 and 10 days prior to start of the extraction process. The cans were filled with 130 ml of stimulant solvent and the heating process of 30 minutes was applied. 30 ml samples was taken after 12 h, 24 h, 48 h and 2 weeks, in order to assess the impact of time of contact between the simulation medium and the polymer layer on the degree of leaching the toxic compounds. pH was set to fall within the 6.5–7.5 range.In order to define the toxicity the acute Microtox® assay was used.

Acute toxicity was assessed by determining the luminescence inhibition of the marine Gram-(–)-bacterium *Vibrio fischeri*, after a 30-min exposure to different samples. The bacteria were purchased in freeze-dried form and activated by

rehydratation with a reconstitution solution (specially prepared nontoxic Ultra--Pure Water) to provide a ready to-use suspension of organisms. The osmolarity of all standard and samples was adjusted to 2% NaCl for the optimal performance. A dilution series of the samples to be analysed was prepared in 2% NaCl. A fixed amount of bacteria was added to the dilution vials. The light emission of this bacterium in contact with different samples and exposure times was measured using the Microtox 500 analyser. The data were processed using the Microtox Omni Software, according to the Basic Test Protocol (81.9%).

3. Results and discussion

Results of studies conducted are presented in Fig. 1. In most cases it can be concluded that the effectiveness of extractant increases with time leading to the reduction of bioluminescence. The water and ethanol extract samples taken after short extraction time (12, 24 h) are an exception. The increase of bioluminescence was noted for these samples proving the hormesis being observed. This resulted from the fact that compounds which penetrated the solutions during the short extraction time were at the concentration levels which favourably influenced the metabolic processes of the test organism. However after 48 h of incubation for both extraction media a significant increase of the toxic effect was observed. This may indicate that in consequence of a longer contact the concentration of xenobiotics in the solution increased or that in consequence of interaction with the simulation medium the initial compounds were transformed (oxidised) into derivative compounds with toxic potential. The highest toxic effect among all extraction media used was noted for 3% acetic acid. Already after 12 h of extraction over 40% increase of the toxic effect was observed as compared to the reference sample, i.e. 3% acetic acid. In other samples a significant (more than 70%) toxic effect occurred. Such an elevated inhibition of bacterial bioluminescence proves that the acetic acid causes the release of a significant amount of small-particle packaging ingredients directly proving the high effectiveness of this medium as a simulation liquid but also showing that consumption of foodstuffs with the pH value lower than 4.5 stored in metal packaging (with polymer layers) may lead to a significant exposure to contact with toxic substances and thus high risk of health consequences.

4. Conclusions

Based on the test results it can be unambiguously concluded that as a result of contact between the inner layer of the packaging and model liquids the lossly bound toxic ingredients of packaging used in the production of protective layers were washed out. In most cases dependless on the extraction time or temperature to which the samples were exposed the reduction of bioluminescence was noted. Based on the data presented in Fig. 1. concluded that the main parameter

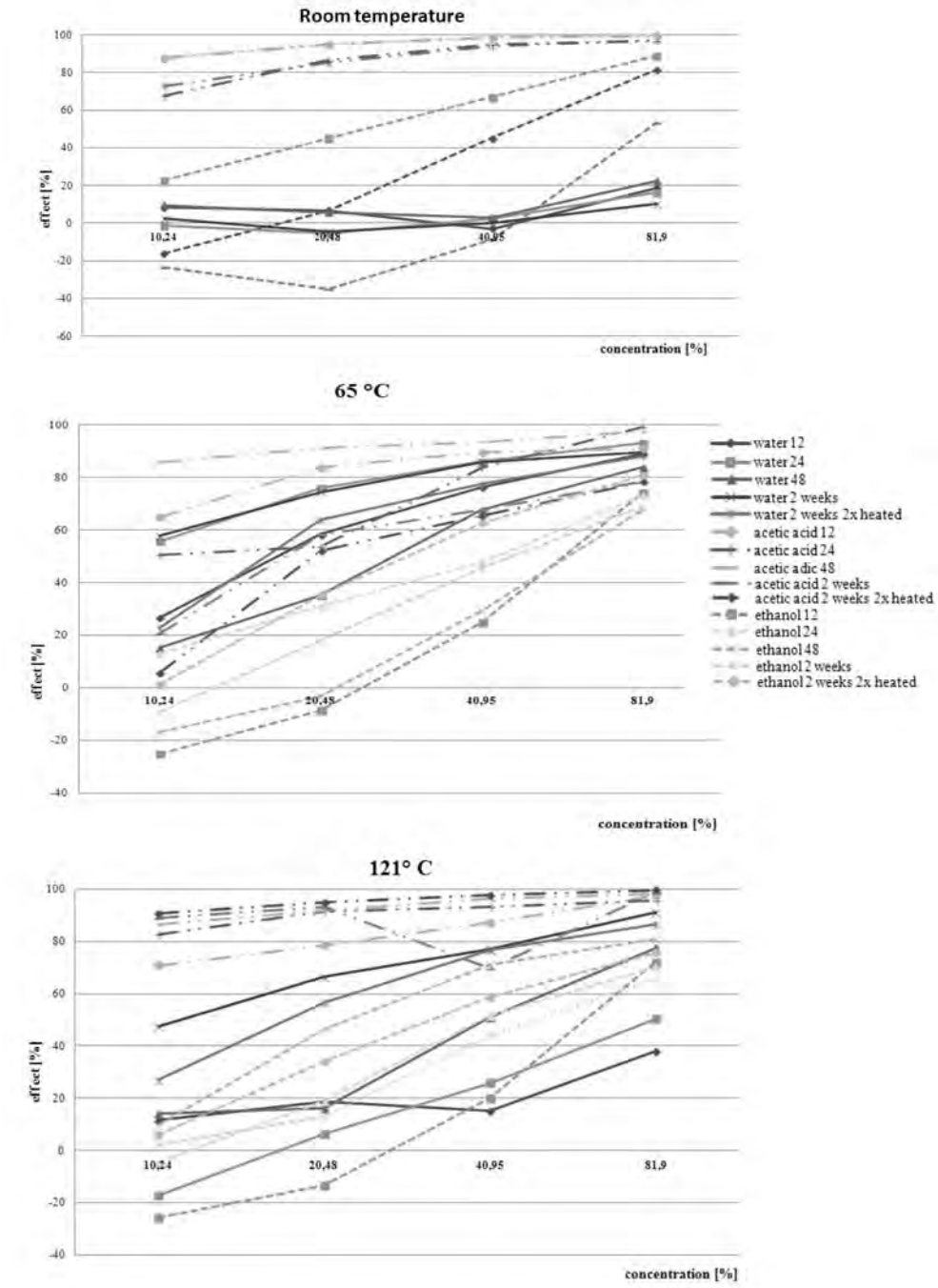


Fig. 1. Graphic representation of the results on toxicity determination of food packages extracts.

responsible for the intensification of the toxic compound migration process is temperature. The time of contact between the extraction medium and the packaging internal layer influences the volume of xenobiotics migration streams. The analysis of results obtained in case of samples exposed to fortnight extraction with double heating process shows that exposing the samples to additional heating does not influence significantly the increase of toxic effect caused by the compounds present in the samples. Based on the tests we may also conclude that consuming foodstuffs with pH lower than 4.5 (in metal packaging with polymer layers) leads to a significant exposure to contact with toxic substances and thus high risk of health consequences in result of migration of toxic products of polymer degradation to foodstuffs.

References

[1] Alabi A., Caballero-Casero N., Rubio S.: *J. Chromatogr. A* **1336** (2014), 23–33.
[2] Grob K., Camus P., Gontard N., Hoellinger H., Joly C., Macherey A. C., Masset D., Nesslany F., Régnier J. F., Riquet A.M., Saillard P., Ribera D.: *Food Control* **21** (2010),763–769.
[3] Cabado A.G., Aldea S., Porro C., Ojea G., Lago J., Sobrado C., Vieites J.M.: *Food Chem. Toxicol*. **46** (2008),1674–1680.
[4] von Saala F.S., Welshons W.V.: *Environ. Res.* **100** (2006), 50–75.
[5] Kudłak B., Szczepańska N., Owczarek K., Mazerska Z., .Namieśnik J.: Endocrine disrupting compounds. Problems and challenges. In: *Emerging Pollutants in the Environment. Current and Further Implications*. M.L. Larramendy (edit.). Rijeka, InTech 2015.
[6] Schaefer A.: *Identification and Quantification of Migrants from Can Coatings. An Approach to Elucidate the Total Migrate below 1000 Da.* Dissertation at Institut für Biochemie und Lebensmittelchemie Abteilung für Lebensmittelchemie, Universität Hamburg, 2004. On-line: http://ediss.sub.uni-hamburg.de/volltexte/2004/2126/pdf/Dissertation.pdf

Classification of juniper-flavoured spirit drinks according to producers by fluorescence spectroscopy

VERONIKA URÍČKOVÁ*, JANA SÁDECKÁ

Slovak University of Technology, Faculty of Chemical and Food Technology, Institute of Analytical Chemistry, Radlinského 9, 812 37 Bratislava, Slovak Republic ✉ veronika.urickova@stuba.sk

Keywords
beverages
fluorescence
multivariate analysis

Abstract
Synchronous fluorescence spectra measured at constant wavelength differences from 10 to 100 nm in the excitation wavelength range 250–350 nm were used for searching the natural grouping among Slovak juniper-flavoured spirit drinks. The best result was achieved using fluorescence spectra recorded at constant wavelength difference 10 nm. Discriminant analysis was used to classify samples considering two types of classification criteria: distinguishing between drinks from different producers or distillates of different geographical indications and others. Discriminant analysis was based on two different data sets: (i) the first five principal components of the principal component analysis performed on the synchronous fluorescence spectra; (ii) the synchronous fluorescence spectra. Regarding different producers, Discriminant analysis based on the principal components and synchronous fluorescence spectra resulted in total 87.5 and 100% correct classification, respectively. Regarding geographical indications, correct classification was 87.5% (principal components) and 100% (synchronous fluorescence spectra).

1. Introduction

Regulation EC No 110/2008) [1] lays down rules on the definition and description of spirit drinks as well as on the protection of geographical indications. A geographical indication identifies a spirit drink as originating in the territory of a country, where a given quality or other characteristic of that spirit drink is essentially attributable to its geographical origin. According to this Regulation, juniper-flavoured spirit drinks are produced by flavouring ethyl alcohol of agricultural origin and/or grain spirit and/or grain distillate with juniper (*Juniperus communis* L. and/or *Juniperus oxicedrus* L.) berries. Some geographical indications include Spišská borovička, Slovenská borovička Juniperus, Slovenská borovička, Inovecká borovička and Liptovská borovička (Slovak Republic).

The identification of the geographical origin of beverages is one of the most important issues in food chemistry. A powerful method for determining the geographical origin is multivariate analysis of the data provided by analytical instruments [2–5].

2. Experimental

2.1 Reagents and chemicals

Commercially available samples from five Slovak producers (code S1–S3) were collected. Different products from the same producer and four (or five) bottles of the same product were sampled. Thus, sample coding was producer, product name, bottle (e.g., S1Z1 means producer S1, product Z, bottle 1). All the samples belonged to the "juniper-flavoured spirit drinks" category, according to EEC Regulations. The alcoholic degree ranged within 35–42% ethanol. The samples were stored at room temperature and analyzed without any prior treatment.

2.2 Instrumentation

Fluorescence spectra were recorded using a Perkin-Elmer LS 50 Luminescence spectrometer equipped with a Xenon lamp. Samples were placed in 10 mm×10 mm×45 mm quartz cell. Excitation and emission slits were both set at 5 nm. Synchronous fluorescence spectra were collected by simultaneously scanning the excitation and emission monochromator in the excitation wavelength range 200–700 nm (with an interval of 1 nm), with constant wavelength differences Δλ between them. Synchronous fluorescence spectra were recorded for Δλ interval from 10 to 100 nm, in steps of 5 nm. Hierarchical cluster analysis, principal component analysisand discriminant analysis were applied to investigate differences between the samples.

3. Results and discussion

The contour plots of total synchronous fluorescence spectra were obtained by plotting the fluorescence intensity as a function of excitation wavelength (λ_{ex}) and wavelength interval Δλ. Hierarchical cluster analysis performed separately on synchronous fluorescence spectra measured at Δλ = 10–100 nm in the excitation wavelength range 250–350 nm was used for searching the natural grouping among drinks S1, S2 and S3. The best result was achieved using fluorescence spectra recorded at Δλ = 10 nm. Principal component analysis was used to examine the similarity among the S1, S2 and S3 samples. Applying principal component analysis to synchronous fluorescence spectra recorded at Δλ = 10 nm in the excitation wavelength range 250–350 nm, the first five principal components explained 99.0% of the total variance, where PC1, PC2, PC3, PC4 and PC5 accounted for 56.8, 28.4, 12.8, 0.8 and 0.2% of the total variance, respectively. Eigen values accounted for by each principal component (PC1–PC5) were 18.2, 9.1, 4.1, 0.2 and 0.1, respectively. The ability of synchronous fluorescence spectra to differentiate between the drinks from three producers was investigated by applying the discriminant analysis to the first five principal components of the

principal component analysis performed on the synchronous fluorescence spectra (Δλ = 10 nm, 250–350 nm). In both the calibration set and the prediction set, 100% correct classification was observed for S1 and S3 samples, while only 67% of S2 samples were properly classified. S2J samples were classified as belonging to S3 group. As this classification was unsatisfactory, we chose to discriminate the samples with discriminant analysis based on the synchronous fluorescence spectra (Δλ = 10 nm) in the range of 250–350 nm with an interval of 5 nm. In this case 100% correct classification was observed for S1, S2 and S3 samples.

4. Conclusions

Discriminant models based on synchronous fluorescence spectra were good for classification of Slovak juniper-flavoured spirit drinks. The synchronous fluorescence spectra recorded at constant wavelength difference 10 nm in the excitation wavelength range 250–350 nm were found to provide the best results, with 100% classification of juniper-flavoured spirit drinks of the three producers. In addition, 100% correct classification was observed between distillates of different geographical indications and other drinks. It appears that fluorescence spectroscopy offers a promising approach for the classification of Slovak juniper drinks as neither sample preparation nor special qualification of the personnel are required, data acquisition and analysis are relatively simple.

Acknowledgments

This research was supported by the Scientific Grant Agency of the Ministry of Education of Slovak Republic and the Slovak Academy of Sciences VEGA No 1/0051/13.

References

[1] European Union (EC) (2008) *Regulation (EC) No. 110/2008 of the European Parliament and of the Council of 15 January 2008 on the definition, description, presentation, labelling and the protection of geographical indications of spirit drinks and repealing Council Regulation* (EEC) No. 1576/89. OJEU L39 (51):1654.
[2] Luykx D.M.A.M., van Ruth S.M.: *Food Chem.* **107** (2008), 897–911.
[3] Saurina J.: *Trends Anal. Chem.* **29** (2010), 234–245.
[4] Drivelos S.A., Georgiou C.A.: *Trends Anal. Chem.***40** (2012), 38–51.
[5] Savchuk S.A., Vlasov V.N., Appolonova S.A., Arbuzov V.N., Vedenin A.N., Mezinov A.B., Grigoryan B.R.: *J. Anal. Chem.* **56** (2001), 214–231.

Comparison of two direct ion sources coupled to a MS system (DART-MS and DIP-MS)

Stefan Viehbeck*, Andreas Hadersdorfer, Frank-Michael Matysik

University of Regensburg, Faculty of Chemistry and Pharmacy, Institute for Analytical Chemistry, Chemo- and Biosensors, Universitätsstraße 31, 93053 Regensburg, Germany
✉ *stefan.viehbeck@chemie.uni-regensburg.de*

Keywords
atmospheric pressure ion sources
direct analysis in real time
direct inlet probe
mass spectrometry

Abstract
In today's industry quick and reliable analysis methods play an important role for quality control. On that account two direct ion sources coupled to a Q-TOF system (Agilent quadrupole time-of-flight 6530) were tested. For one, the "Direct Inlet Probe (DIP-APCI)" ion source was used to analyze small amounts of solid or liquid samples. The concept of the ion source is based on an APCI source for mass spectrometry, however, in this case the samples are introduced directly via a pushrod. A similar system is the "Direct Analysis in Real Time (DART)" ion source. In addition to the analysis of solid and liquid samples without preseparation, this ion source offers the possibility to scan the surface of a sample. A method for industrial sample analysis was developed using DIP-APCI-/DART-Q-TOF-MS.

1. Introduction

In the last decade, attention towards so-called "direct ion sources" in relation to mass spectrometry has increased. This emerging new ionization technique enables direct analysis of untreated samples without preliminary separation. One of the best known examples of this sector is the matrix-assisted laser desorption [1]. Comparable methods, which have a wider field of applications, such as "Direct Analysis in Real Time – IonSense" (DART) [2] and "Direct Inlet Probe –SIM GmbH" (DIP) ion sources were tested for a variety of industrial samples.

2. Experimental

The samples were introduced directly into the ion sources without any sample preparation or preseperation. In this case the delamination of an e-coat from a metal plate was examined. For the analysis with the DART ion source (Fig. 1) the sample was introduced directly into the excited and heated helium stream. As ideal working temperature for easily volatile components the gas stream of the DART was set to 350 °C. The ionization takes place through penning ionisation [3]

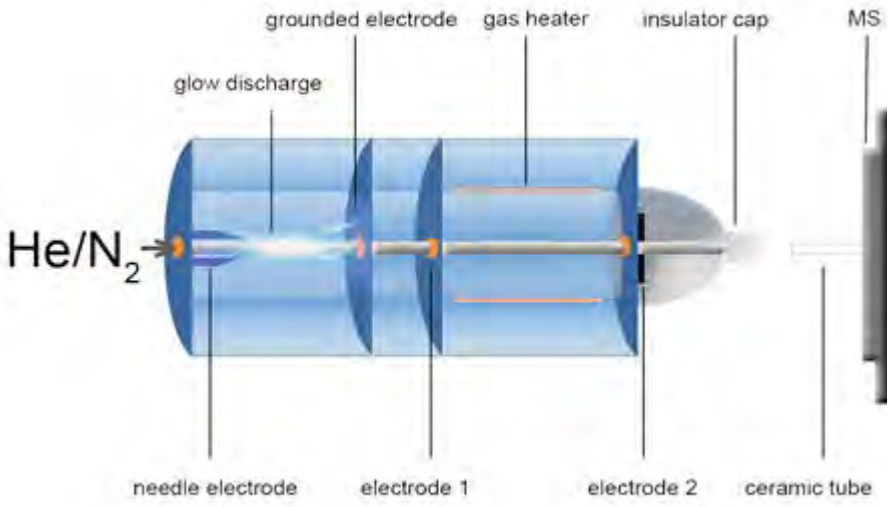


Fig. 1. DART ion source – setup, the helium can be heated up to 500 °C and is exited to metastable state through glow discharge.

Fig. 2. DIP ion source – setup, modified APCI ion source with a pushrod, the analytes are vaporized and charged through corona discharge.

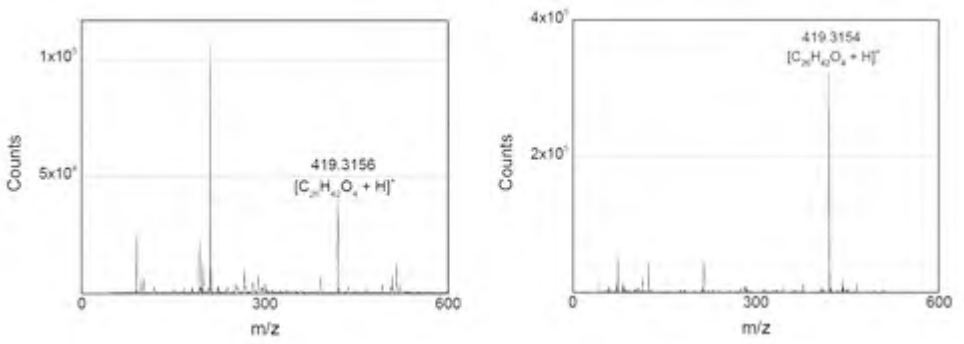


Fig. 3. DART-Q-TOF (left) and DIP-APCI-Q-TOF (right) mass spectra from the surface of the e-coat. The plasticizer didecylphthalate ($C_{28}H_{26}O_4$) was identified as cause for the detachment.

and proton exchange [4]. On the other hand, for the analysis with the DIP ion source, small pieces of the sample were scraped off the surface and added to the crucible on top of the push rod from the DIP (Fig. 2) [5]. The temperature setting of the ion source was set to a gradient to ensure an ideal evaporation of the sample. The first temperature point was set to 300 °C and held for one minute, the second one to 350 °C for two minutes before reaching the maximum of 400 °C for one minute. The heating rate is variable and was set to 2 °/s. All volatile components are ionized through corona discharge similar to the ionization process of an APCI ion source [6].

3. Results and discussion

In both mass spectra (Fig. 3) a signal appears with the *m/z* ratio of 419. After generating the molecular formula the signal was identified as protonated didecylphthalate $[C_{28}H_{26}O_4+H]^+$. Contamination or migration of plasticizers to the layer between the substrate and a varnish are often the cause for irregularities and delamination. Thus both ion sources were able to ionize the molecules responsible for the detachment and therefore were detected by the Q-TOF-MS. However an advantage of the DIP ion source is the temperature gradient, which generates a noticeable preseparation because of the different evaporation temperatures of the molecules in the sample matrix. Regarding the DART ion source the advantage can be seen in the flexibility of the samples, which can be analyzed. Even the surface of larger samples from the automotive industry can be screened with the DART ion source.

4. Conclusions

With both ion sources the working steps and the required time for sample analysis were significantly reduced, due to the elimination of sample preparation or preseparation. Consequently, laboratories can increase their capacity and reduce the workflow by applying the developed method.

Acknowledgments

I would like to express my gratitude to all of the department faculty members for their help. I am also grateful to my former supervisor Dr. A. Hadersdorfer and to Prof. F.-M. Matysik for initiating my studies in this field of research and for their support.

References

[1] Karas M., Bachmann D., Hillenkamp F.: *Anal. Chem.* **57** (1985), 2935–2939.
[2] Cody R. B., Laramée J. A., and Durst H. D.: *Anal. Chem.* **77** (2005), 2297–2302.
[3] Penning F. M.: *Die Naturwissenschaften* **15** (1927), 818.
[4] Cody R. B.: *Anal. Chem.* **81** (2009), 1101–1107.
[5] Krieger S., Hayen H., Schmitz O.: *Anal. Bioanal. Chem.* **405** (2013), 8337–8345.
[6] Carroll D. I., Dzidic I., Stillwell R. N., Haegele K. D., Horning E. C.: *Anal. Chem.* **47** (1975), 2369–2373.

Examination of excretion of ibuprofen and its metabolites in horses

Emilia Waraksa[a, b, *], Marzena Wójtowicz[b], Dorota Kwiatkowska[b], Jacek Namieśnik[a]

[a] *Department of Analytical Chemistry, Faculty of Chemistry, Gdańsk University of Technology, Narutowicza Street 11/12, 80-233 Gdańsk, Poland* ✉ *emilia.waraksa@op.pl*
[b] *Institute of Sport, Trylogii Street 2/16, 01-982 Warszawa, Poland*

Keywords
doping
GC-MS
ibuprofen
horse
urine

Abstract
Analytical procedure based on application of GC-MS at the step of detection, identification and quantitation of ibuprofen and detection of its metabolites (1-hydroxyibuprofen and carboxyibuprofen) in horse urine by selected ion monitoring mode after sample preparation covering extraction at pH = 3–4 and methylation has been used. The ions at *m/z* 161, 220 (methylated ibuprofen), 178, 119 (methylated 1-hydroxyibuprofen) and 145, 264 (methylated carboxyibuprofen) was monitored to indicate the presence of investigated compounds in horse urine. The method was successfully applied to urine samples taken from two horses that had been given a single oral dose of 0.9 and 1.1 mg/kg of ibuprofen respectively. The results of excretion of ibuprofen and its metabolites were reported.

1. Introduction

Non-steroidal anti-inflammatory drugs are widely used in equine medicine. These drugs are the main doping agents found in horse samples [1]. Non-steroidal anti--inflammatory drugs are often misused in competing horses in order to mask signs of pain or inflammation. Due to this fact non-steroidal anti-inflammatory drugs are prohibited substances in competition by different veterinary regulations [2–6].

Ibuprofen, 2-(4-isobutylphenyl)propionic acid, is a muscarinic antagonist extensively used in the treatment of acute and chronic pain and many rheumatic and musculoskeletal disorders and sometimes used in competing horses to hide their illnesses [2, 7, 8]. Due to this fact, ibuprofen is listed on FEI Equine Prohibited List as a controlled substance. Because of ibuprofen widespread use in human and veterinary medicine, there have been reports describing the metabolism and pharmacokinetics of ibuprofen in man. A gas GC-MS procedure for the toxicological detection of ibuprofen and its two metabolites (1-hydroxyibuprofen and carboxyibuprofen) in human urine after extraction at pH = 5 and methylation has been already applied [9]. However, several factors may lead to differences in drug metabolism between horses and humans [10]. Thus, attempts were also made to

examine of elimination of ibuprofen in horses. Gas chromatography with flame ionization detector procedure for detection of ibuprofen in horse urine after extraction at pH = 2 and silanization has been applied [11]. However, two main metabolites of ibuprofen were not silylated under the outlined conditions. Thus, there is a lack of reports of excretion of ibuprofen with the use of GC-MS and examination of horse metabolism. The aim of our study is to prove that two main human urine metabolites of ibuprofen can be detected in horses' urine samples despite differences in diet and construction of digestive system in both species which indicates that the metabolism of ibuprofen is the same in the case of humans and horses. The second purpose is examination of excretion of ibuprofen and its metabolites in horses with the use of GC-MS which has not been reported yet.

2. Experimental

2.1 Reagents and chemicals

The compounds were supplied by the following manufactures: ibuprofen (Sigma--Aldrich), methyl iodide (Sigma-Aldrich), ethyl acetate (Rathburn Chemicals, Scotland). Anhydrous potassium carbonate, anhydrous sodium sulphate, diethyl ether, sodium hydrogencarbonate, methanol, hydrochloric acid 37% and water were obtained from Avantor Performance Materials (Poland). Preazone was synthesized in Doping Control Laboratory in Köln.

2.2 Sample preparation

The urine samples were taken from two horses (H1 and H2) before single oral administration of 0.9 mg/kg and 1.1 mg/kg ibuprofen respectively and till five days after the administration. Scheme of horse's urine sample preparation for ibuprofen and its metabolites is shown in Fig. 1.

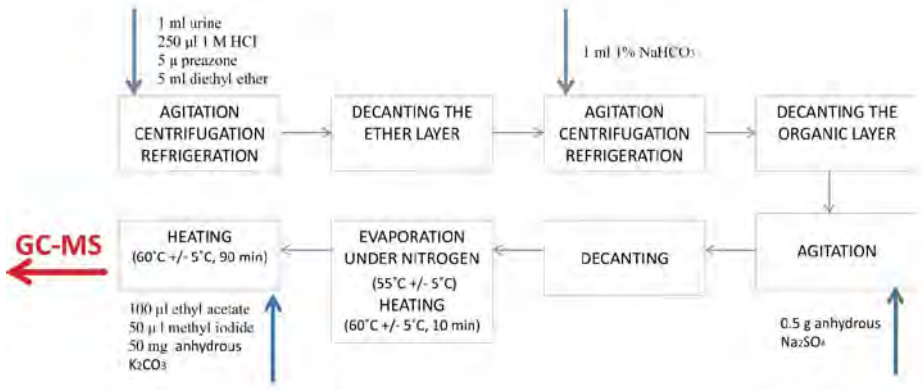


Fig. 1. Scheme of horse's urine sample preparation for ibuprofen and its metabolites detection.

2.3 Instrumentation

The analyses were performed on a 6890 GC gas chromatograph coupled to a 5975B MS mass spectrometer (both Agilent Technologies) applying the following parameters: column Agilent 19091Z-002 HP-1MS (25 m × 200 μm i.d., 0.11 film thickness), constant flow of helium carrier gas (1.3 ml/min); initial column temperature 130 °C (1 min) and 6 °C/min ramp to 310 °C (1 min); splitless module injection at 280 ºC. Selected ion monitoring (SIM) was used.

3. Results and discussion

Ibuprofen and its two metabolites, 1-hydroxyibuprofen and carboxyibuprofen, were identified by MS after methylation and GC separation. Characteristic ions at *m/z* 161, 220 (methylated ibuprofen), 178, 119 (methylated 1-hydoxyibuprofen) and 145, 264 (methylated carboxyibuprofen) were used to indicate the presence of ibuprofen and its metabolites in horse urine. The quantitative analysis in the excretion study of ibuprofen was based on the ions at m/z 161 and 183 (for ISTD). Representative chromatograms of drug-free horse urine sample and urine sample collected 46 h (56 h resp.) after administration of ibuprofen are shown in Fig. 2.

Mass spectra of ibuprofen from quality control sample and ibuprofen, 1-hydroxyibuprofen, and carboxyibuprofen from horse urine sample are presented in Fig. 3.

The concentration of ibuprofen in all collected samples was recalculated with the use of a specific gravity correction. Excretion curves ibuprofen and its metabolites both horses H1 and H2 are shown in Fig. 4. The results obtained for two horses which were given single oral dose (0.9 mg of ibuprofen for horse H1 and 1.1 mg for horse H2) were similar to each other. Ibuprofen was detected from 23 to 100 h while 1-hyroxyibuprofen and carboxyibuprofen were detected from 40 to 80 h in both horses' urine samples. Differences between periods of time in which ibuprofen, 1-hydroxyibuprofen and carboxyibuprofen may be detected are not significant. Although periods of time in which metabolites may be detected are not longer than in the case of parent compound, they may be also applied to indicate and monitor the use of ibuprofen. The maximum concentration of ibuprofen was found in the 47 h urine with level of 4.0 μg/ml for the horse H1 and 56 h urine with level up to 1.2 μg/ml for the horse H2. The maximum ratio of peak areas for 1-hyroxyibuprofen and ISTD as well as peak areas for carboxyibuprofen and ISTD was found in the 47 h urine for the horse H1 and 56 h urine for the horse H2. It is worth mentioning that regardless of dose (0.9 mg/kg for horse H1 and 1.1 mg/kg for horse H2), the excretion curves were very similar. The differences in the shape of the presented excretion curves can be related to the inter-and intra-individual variability in excretion. It should be also mentioned that the same diet for the horses was maintained during the excretion study.

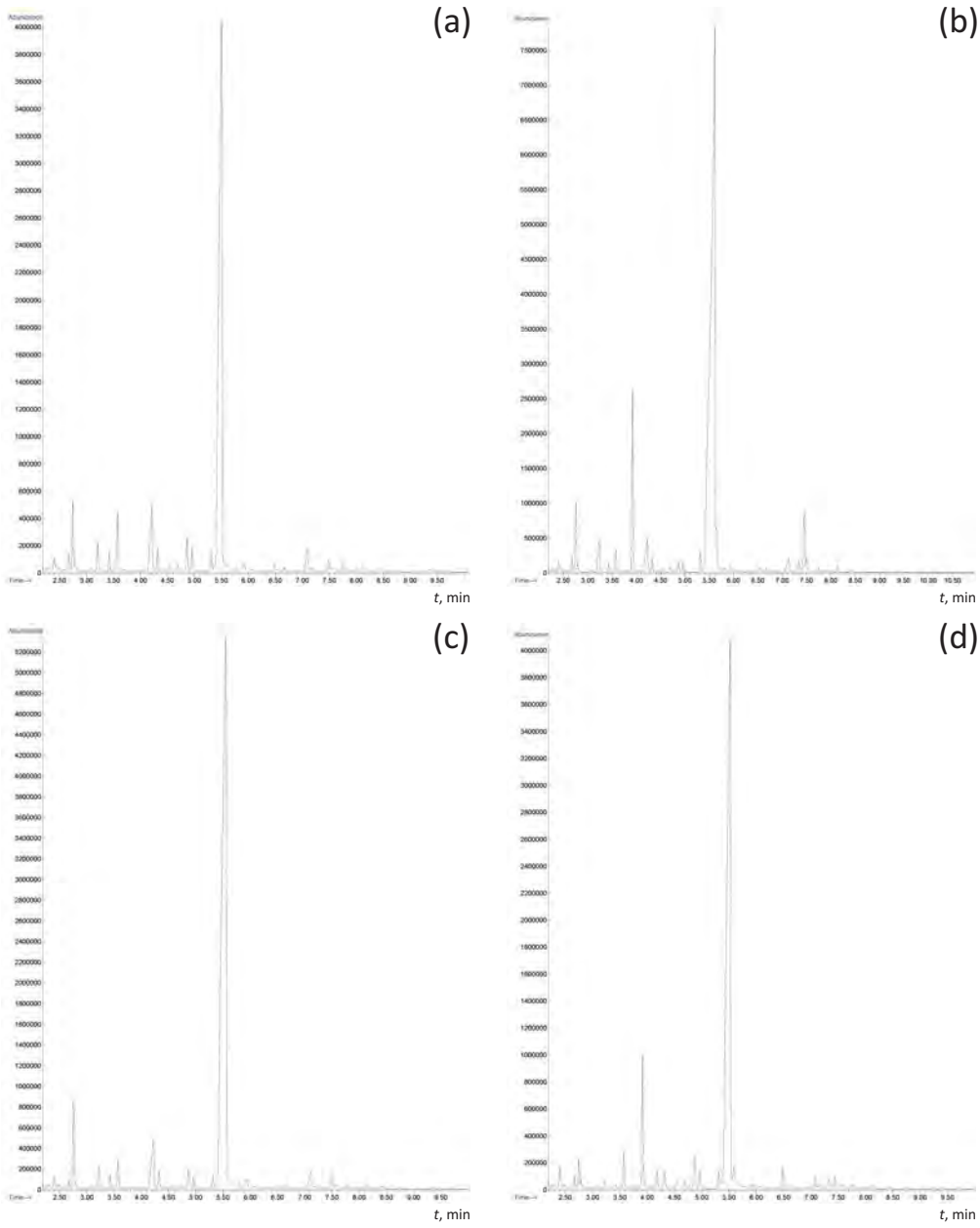


Fig. 2. Chromatograms obtained during analysis of samples of extract from horse urine (a) drug free horse H1's urine sample, (b) horse H1's urine sample obtained 46 h after administration, (c) drug free horse H2's urine sample, (d) horse H2's urine sample obtained 56 h after the administration.

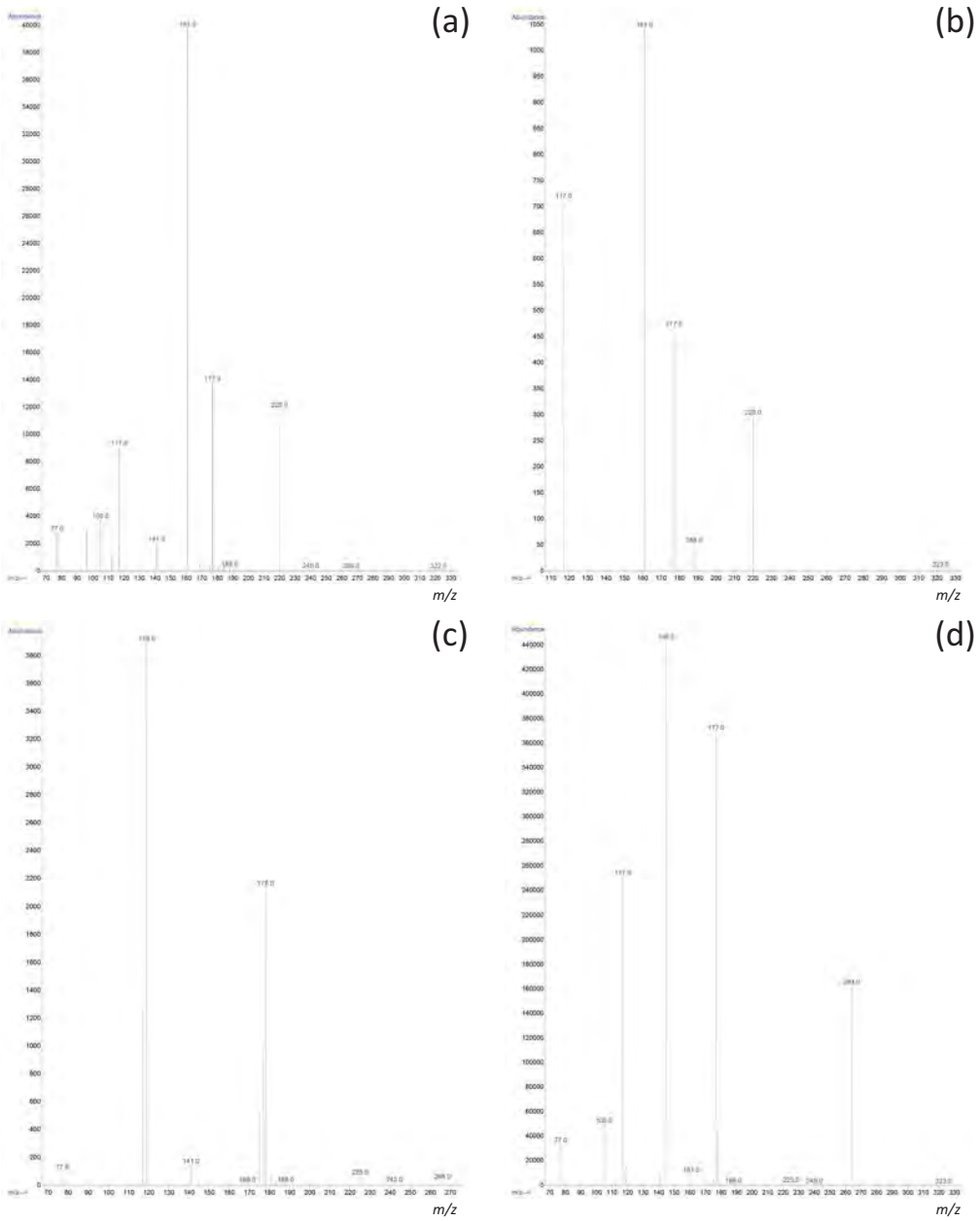


Fig. 3. Mass spectra of (a) ibuprofen from quality control sample (ibuprofen concentration of 50 ng/ml), (b) ibuprofen from horse H1's urine sample collected 46 h after the administration, (c) 1-hydroxyibuprofen from horse H1's urine sample collected 46 h after the administration, (d) carboxyibuprofen from horse H1's urine sample collected 46 h after the administration.

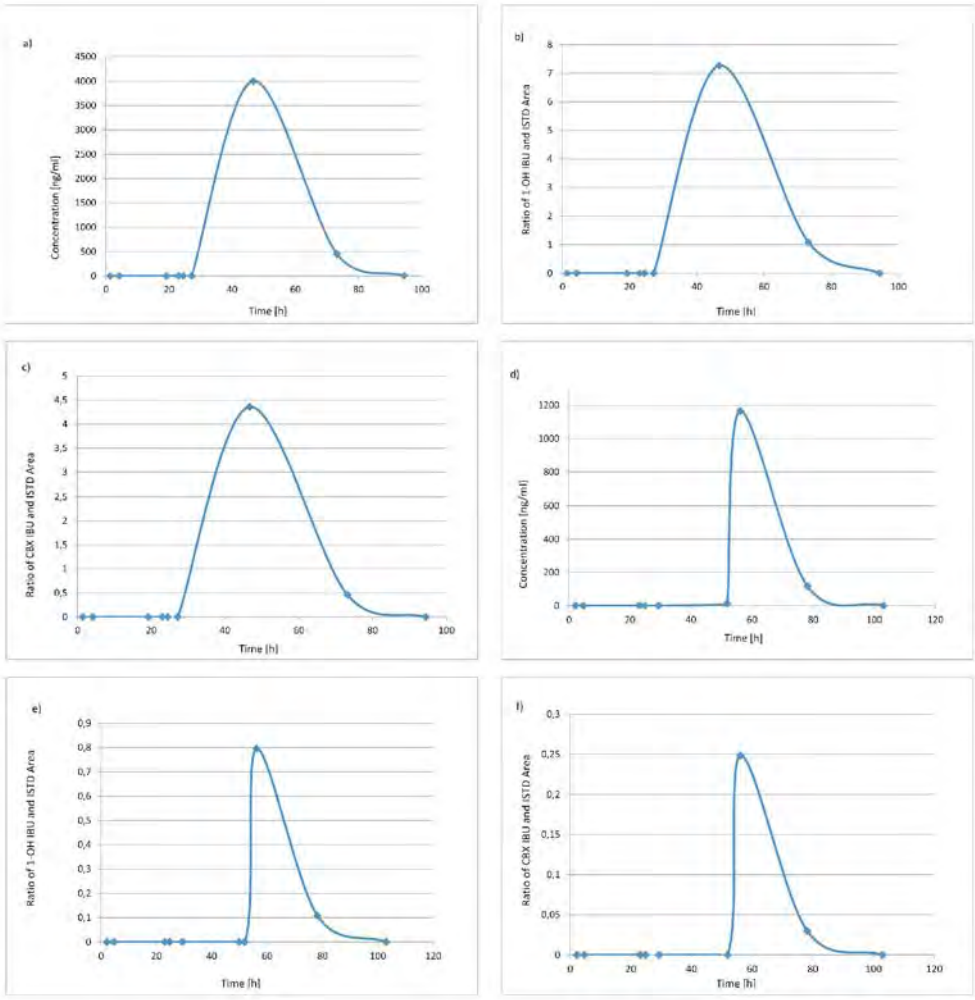


Fig. 4. Excretion curve of (a) ibuprofen in horse H1, (b)1-hydroxyibuprofen in horse H1, (c) carboxyibuprofen in horse H1, (d) ibuprofen in horse H2, (e) 1-hydroxyibuprofen in horse H2, (f) carboxyibuprofen in horse H2.

4. Conclusions

The GC-MS procedure method of determination of ibuprofen has been successfully applied to the excretion study in horses. The qualitative methods for 1-hyroxyibuprofen and carboxyibuprofen have been also used for excretion studies purposes. It was proven that although there are differences in drug metabolism in horses and humans, two metabolites of ibuprofen could be detected in both, human and horse urine samples According to significant importance of the elimination time of doping agents in equine antidoping control, the excretion study of ibuprofen and its two metabolites has been investigated.

Human excretion time of ibuprofen and its metabolites was investigated in other studies and it was showed that period of time in which carboxyibuprofen may be detected is much longer than in the case of parent compound [8]. Taking into account results obtained for human excretion of ibuprofen and carboxyibuprofen, it had been expected that horse excretion time of carboxyibuprofen would be longer than excretion time of ibuprofen. However, it has been proved in this study that excretion time of ibuprofen, 1-hydroxyibuprofen and carboxyibuprofen in horses is similar to each other. It leads to conclusions that although periods of time in which metabolites may be detected are not longer than in the case of parent compound, they may be also applied to indicate and monitor the use of ibuprofen According to the obtained results, ibuprofen is detected from 23 to 100 h in horse urine samples after the administration. However in another study, it was reported that ibuprofen was detected with the use of GC-FID from a few minutes to 28 h after the administration. Differences in obtained results could be due to differences in horses' rase and diet. In this study samples were collected from urine in naturally urination process while in the previous study, the catheter was used as evidenced by sampling frequency [10].

Acknowledgments

The author thanks Eleonora Waraksa, who is the owner of horses, for permission to carry out the research.

References

[1] González G., Ventura R., Smith A.K., De la Torre R., Segura J.: *J. Chromatogr. A* **719** (1996), 251–264.
[2] www.fei.org
[3] Kim J.Y., Kim S.J., Paeng K.J., Chung B.C.: *J. Vet. Pharmacol. Therap.* **24** (2001), 315–319.
[4] Takeda A., Tanaka A.H., Shinohara T., Ohtake I.: *J. Chromatogr. B* **758** (2001), 235–248.
[5] Azzouz A., Ballesteros E.: *J. Chromatogr. B* **891–892** (2012), 12–19.
[6] Maurer H.H.: *J. Chromatogr. B* **733** (1999), 3–25.
[7] Yilmaz B., Erdem A.F.: *J. AOAC Int.* **97** (2014), 415–420.
[8] Ferrando-Climent L., Collado N., Buttiglieri G., Gros M., Rodriquez-Roda I., Rodriquez-Mozaz S., Barceló D.: *Sci. Total Environ.* **438** (2012), 404–413.
[9] Maurer H.H.,Kraemer T., Weber A.: *Die Pharmazie* **49** (1994), 148–155.
[10] Scarth J.P., Teale P., Kuuranne T.: *Drug Test. Analysis* **3** (2011), 19–53.
[11] Evans J.A., Lambert M.B., Miller J.: *Br. J. Sports. Med.* **10** (1976), 124–127.

Application of bioassays in studies on the impact of pharmaceuticals on the environment

Monika Wieczerzak*, Błażej Kudłak, Jacek Namieśnik

Gdańsk University of Technology, Faculty of Chemistry, Department of Analytical Chemistry, 11/12 G. Narutowicza Str., 80-233 Gdańsk, Poland ✉ monwiecz@student.pg.gda.pl

Keywords
drug residues
ecotoxicity
Microtox®
pharmaceuticals' interaction

Abstract
Pharmaceuticals (diclofenac sodium salt, oxyteracycline hydrochloride, fluoxetine hydrochloride, and chloramphenicol), were mixed with each other at different ratios of EC_{50} and tested using Mictotox®. Results obtained show the highest toxicity exhibited by mixtures containing diclofenac.

1. Introduction

Studies on the state of the environment often focus on the determination of quantitative and qualitative composition of collected samples (using, for example. HPLC, GC-MS and various methods of sample preparation). Additionally, results are complemented by physical and chemical parameters such as pH, COD. Modern approach more often presented in the literature connects together the information obtained by means of biological and instrumental methods, using for this purpose bioanalytical tools e.g. bioassays.

Pharmaceuticals are an example of frequently tested compounds, both by instrumental and biological methods yet their environmental fate still remains largely unexplored. Drug interactions are often desirable particularly in medicine, when treatment of a patient requires using a mixture of drugs in specific doses to produce the adequate therapeutic effect. Unfortunately, interactions between drugs in the environment are not as desirable, despite the fact that drugs are present in the environment at low concentration levels [1]. Pharmaceuticals are present in the environment in a mixture of compounds, which are not inert towards each other, may contribute to the increase (phenomenon of synergism) or decrease (phenomenon of antagonism) of overall toxicity [2].

In Poland an average citizen buys and consumes about 29 packs of drugs what places Poland in fifth place in Europe right after France, Germany, Italy, and the UK. Consumption of the most popular drugs reaches as much as several hundred tons; for example, annual consumption of ibuprofen in Germany in 2000 amounted to 300 tons, 162 tons in England, Poland 58 tons and 25 tons in Switzerland [3].

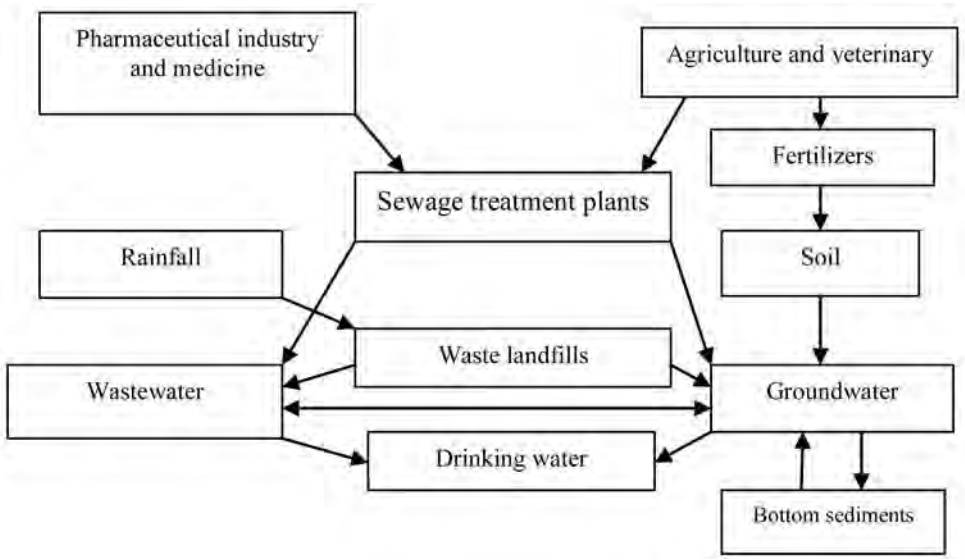


Fig. 1. Pathways of drugs in the environment [4].

Fig. 1. shows the pathways and the main sources of pharmaceuticals in the environment. Unfortunately pharmaceuticals are not completely eliminated in traditional wastewater treatment technologies and in addition, medical waste and other unused pharmaceuticals from landfills, often poorly sealed and protected, reach ground water due to infiltration and leakages [4].

Drug residues in the environment drew the attention of the scientific community when it became clear that present even at low concentration levels in the environment (often few ng/L or μg/L) can cause adverse effects (e.g. endocrine disruption) [5].

Toxic effects caused by the drug residues can vary and depend on the affected organism and the type of drug. Scientific evidence indicates that for example ibuprofen inhibits the growth of gram-positive bacteria as it does not affect the growth of colonies of gram-negative bacteria, and have an anti-fungal properties against a certain kind of dermatophyte fungi [4, 6].One of the serious consequences of drug abuse and their accumulation in the environment is drug resistance and the emergence of pathogens resistant to antibiotics, which seriously hampers the treatment diseases [4].

Chemicals with proven therapeutic effect undergo many phases of clinical trials designed to detect whether a given compound will be safe for the patient. Unfortunately the studies of environmental fate of pharmaceutical are rare.

Studying the environmental fate of pharmaceutical residues one have to take into account that these compounds are present in the mixture and do not remain neutral to each other. Effect of the mixture of compounds may occur greater (synergism) or lower (antagonism) than individually compounds [7].

In the present work, the influence of mixtures of four drugs against the bacterium *Vibrio fischeri* has been measured. Gram(–) bacilli *Vibrio fischeri* occur in salt waters, and bioluminescence is a natural result of their metabolic processes. The decrease or increase in the bioluminescence of bacterial suspension after a period of incubation with the test sample is the basis of the test Microtox® used in this study. After the incubation period follows reading of the level of bioluminescence and calculating the EC_{50} parameter by the software [8].

Tested pharmaceuticals namely: diclofenac (sodium salt), oxyteracycline hydrochloride, fluoxetine hydrochloride and chloramphenicol are widely used in various therapeutic treatments. Oxytetracycline hydrochloride derived from the tetracyclines, which exhibit bacteriostatic and bactericidal properties against both G(+) and G(–) bacteria, is used as a veterinary and aquaculture agents [9]. Chloramphenicol also has bac-teriostatic properties against the G(+) and G(–), but due to the undesirable side effects is used only in case of life-threatening infections such as: cases of plague or tuberculosis. In small amounts in the form of ointments or suspensions can be applied local to the skin, eye or ear [10]. Diclofenac is a drug from the group of non-steroidal anti-inflammatory drugs mainly used to treat musculoskeletal pain and chronic pain [11]. Fluoxetine hydrochloride is used in anti-depressant therapy and belongs to the group of selective serotonin reuptake inhibitors [12]. In Table 1 information about concentration levels of select group of pharmaceuticals in the environment is summarized. The data indicates that there is a risk of adverse effects of those compounds' presence.

2. Experimental

2.1 Reagents and chemicals

The Microtox® test reagent (lyophilized/freeze dried *Vibrio fischeri*), osmotic adjustment solution (OAS), reconstitution solution (RS), and diluent were purchased from Modern Waters (USA).

Table 1
Literature study on occurrence and concentration of selected pharmaceuticals in the environment.

Analyte	Concentration in environment [ng/L]	Sample matrix/sample location	Ref.
Diclofenac	10–55	Surface water samples (river Vantaa), Finland	[13]
Chloramphenicol	<2–15	River water samples (Taff and Ely rivers), South Wales, UK	[14]
Oxytetracycline	377000±142000	Waste water, China	[15]
Fluoxetine	0.5–43.2	Stream water samples, USA	[16]

Model substances selected for the study: diclofenac (sodium salt) (CASN [15307-79-6]), chloramphenicol (CASN [56-75-7]), oxytetracycline hydrochloride (CASN [2058-46-0]), fluoxetine hydrochloride (CASn [56296-78-7]) were purchased form Sigma-Aldrich.

2.2 Instrumentation

The study was conducted using Microtox® analyzer model 500 (M500). Apparatus is equipped with 30 incubation wells as well as reagent (bacterial suspensions) and read wells. Temperatures are assigned to the corresponding type of performed test (in this case acute toxicity test) and the internally maintained at 5.5±1 °C for reagent well and 15± 0.5°C for both the incubator part and the read well.

2.3 Microtox® Acute Toxicity Assay

In order to determine the EC_{50} values for four selected drugs the range screening tests were performed according to standard protocol contained in the Microtox Users Manual with small modifications. Lyophilized reagent was hydrated with reconstitution solution and maintained at 5.5±1 °C. 100 µl of prepared in diluent cell suspension was added into the cuvettes in every other row. Subsequently into vials with bacteria, a pre-made standard dilution of the samples was added.

Each test was performed with fourteen dilutions of each standard. After narrowing the search range for the EC_{50} additional tests were performed for each standard in three replicate in four dilutions. In order to determine whether the addition of one substance to another would change the toxic effect, concentrated solutions of the compounds were prepared. Test mixtures were prepared in such a way that the compounds were present in an appropriate ratio respectively 100% of first model substance EC_{50} and 66% and 33% of EC_{50} of the second substance tested and the second substance with a reduced effect to 33% and 66% of EC_{50}.

To produce a suitable osmotic pressure (above 2%) OAS was added to each test vial. Sample incubation time was 30 min.

3. Results and discussion

Results were plotted in the dose-response curve, which was constructed by plotting the percent of bioluminescence change against the pharmaceuticals concentrations. EC_{50} results obtained for each drug against *Vibrio fischeri* are shown in Table 2.

In Fig. 2 are presented results obtained for the mixtures of diclofenac and fluoxetine hydrochloride, which showed the highest toxicity towards *Vibrio fischeri.* Toxicity was elevated when compared to controls of individual compounds prepared at 100%, 66% and 33% of the EC_{50} (respectively 0.037, 0.024, 0.012 [mmol/dm^3] for fluoxetine hydrochloride and 0.72, 0.47, 0.24 [mmol/dm^3]

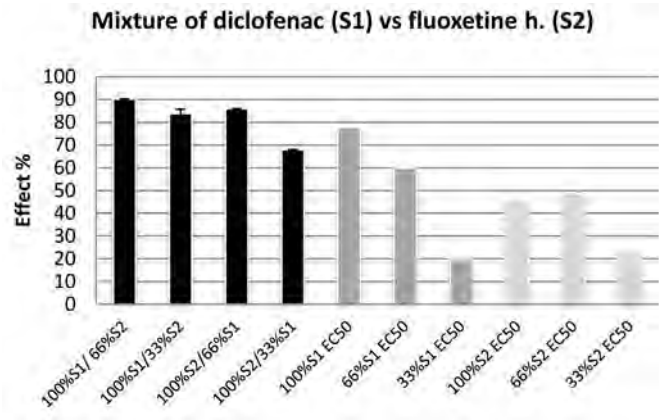


Fig. 2. The results obtained for the mixture of diclofenac and fluoxetine hydrochloride.

Table 2
EC_{50} valuesfor the tested pharmaceuticals.

Analyte	EC_{50} [mmol/dm^3]
Oxytetracycline hydrochloride	0.11
Fluoxetine hydrochloride	0.037
Diclofenac (sodium salt)	0.72
Chloramphenicol	0.92

for diclofenac). The most toxic combination is proven at the ratio of 100% EC_{50} of diclofenac and 66% EC_{50} of fluoxetine.

Mixtures of diclofenac and fluoxetine hydrochloride were found to be more toxic than mixtures of antimicrobial compound e.g. chloramphenicol and oxyteracycline, for which the effect did not exceed 60% of bioluminescence inhibition.

4. Conclusions

Among the individual tested pharmaceuticals, the ranking of toxicity was fluoxetine hydrochloride > oxytetracycline hydrochloride > diclofenac (sodium salt) > chloramphenicol. Studies have shown the highest toxicity values are obtained for mixtures of diclofenac and fluoxetine.

The results obtained together with the plan end studies will contribute to the knowledge about the environmental impact of pharmaceuticals but still there is a need to examine numerous mixtures of pharmaceuticals and inorganic ions in order to extend and verify existing knowledge. Also in conjunction with information obtained with the use of methods for the determination of pharmaceuticals in

the environment contribute to better illustrate processes that occur in ecosystems.

Acknowledgments

This research is funded by the Polish National Science Centre No.UMO-2013/09/N/NZ8/03247.

References

[1] Hernando M.D., Mezcua M., Fernández-Alba A.R., Barceló D.: *Talanta* **69** (2006), 334–342.
[2] Wieczerzak M., Kudłak B., Namieśnik J.: C*rit. Rev. Anal. Chem.* **45** (2015), 131–155.
[3] Dębska J., Kot-Wasik A., Namieśnik J.:*Crit. Rev. Anal. Chem.* **34** (2004), 51–67.
[4] Halling-Sørensen B., Nors Nielsen J.S., Lanzky P.F., Ingerslev F., Holten-Liitzhofl H.C., Jorgensen S.E.: *Chemosphere* **36** (1998), 357–393.
[5] Kudłak B., Namieśnik J.: *Crit. Rev. Anal. Chem.* **38** (2008), 242–258.
[6] Elvers K.T., Wright S.J.L.: *Lett. Appl. Microbiol.* **20** (1995), 82–84.
[7] Wieczerzak M., Kudłak B., Namieśnik J.: *Crit. Rev. Anal. Chem.* **42** (2015), 131–155.
[8] Marugán J., Bru D. Pablos C., Catalá M.:*J. Hazar. Mat.* **213–214** (2012), 117–122.
[9] Gao C., Liu Z., Chen J., Yan Z.: *Luminescence* **28** (2013), 378–383.
[10] Cho Y.K., Huang W., Kim G.Y., Lim B.S.: *Curr. Eye Res.* **38** (2013), 9–17.
[11] Ryshetti S., Gardas R.L.,Tangeda S.J.: *J. Chem. Thermo.* **82** (2015), 125–133.
[12] Yellamula N.R., Appapurapu A.K., Praveen T., Reddy K., Banji D., Appapurapu A.K., Deepthi P.N.: *The Pharma Innovation Journal* **3** (2013), 1–9.
[13] Vieno N.M., Härkki H., Tuhkanen T., Kronberg L.: *Environ. Sci. Technol*. **41** (2007), 5077–5084.
[14] Kasprzyk-Hordern B., Dinsdale R. M., Guwy A. J.: *Water Research* **42** (2008), 3498–3518.
[15] Li D., Yang M., Hu J., Ren L., Zhang Y., Li K.: *Environ. Toxicol. Chem.* **27** (2008), 80–86.
[16] Schultz M.M., Furlong E.T, Kolpin D.W., Werner S.L., Schoenfuss H.L., Barber L.B., Blazer V.S., Norris D.O., Vajda A.M.: *Environ. Sci. Technol.* **44** (2010), 1918–1925.

The comparison of vodka, whisky and moonshine by using the electronic nose based on the technology of ultra-fast gas chromatography

Paulina Wiśniewska*, Magdalena Śliwińska, Tomasz Dymerski, Waldemar Wardencki, Jacek Namieśnik

Department of Analytical Chemistry, The Chemical Faculty, Gdansk University of Technology, 11/12 Narutowicza St., 80-233 Gdańsk, Poland ✉ *p.m.wisniewska@gmail.com*

Keywords
e-nose
statistics
vodka
whisky

Abstract
Vodka and whisky are produced from grain distillates. Formerly these alcohols were very often counterfeited, by the made in home conditions spirit drink called moonshine, which is obtained by distilling a mash from the alcoholic fermentation. This study presents research aimed at checking the usefulness of the electronic nose using ultra-fast gas chromatography for the differentiation of whisky, vodka and moonshine. 12 samples of different spirits beverages were analyze by Heracles II electronic nose. Data were analyzed by the following chemometric methods: PCA – Principal Component Analysis, DFA – Discriminant Function Analysis, SIMCA classification – Soft Independent Modeling of Class Analogies, SQC – Statistical Quality Control.

1. Introduction

Alcohols produced from grains are one of the most widely consumed beverages. Their group includes vodka and whisky. For the production of vodka there are used commonly rye distillates, or mixed e.g. from rye, barley and wheat. While in the case of some whisky for example the American whisky there are used, in addition to the aforementioned distillates, also distillates of corn. Vodka does not contain any additives other than water. While whisky includes water and caramel for the colouration of whisky [1–4]. In addition, in contrast to the vodkas, whisky is subjected to aging process where gains additional flavor and aroma.

Formerly these alcohols were very often counterfeited, by the made in home conditions spirit drink called moonshine. It is obtained by distilling a mash from the alcoholic fermentation. Depending to taste of producers, it can be purified with activated charcoal gaining the taste of commercial spirit, or enriched by the aging. As the drink produced in home conditions it can have many impurities, distinguishing it from the spirit or vodka. Some of the contaminants of moonshine,

especially the lighter alcohols and ketones, are also in the noble alcohols produced by traditional methods (cognac, whisky, grappa) and it may have a positive impact on its taste [5–7].

So far, there are no studies which have been conducted to compare the vodka and whisky to moonshine, which can be used for their counterfeiting. Studies carried out on whisky and vodkas are usually conducted using a one-dimensional gas chromatography [8–15] or two-dimensional gas chromatography [16] and related to the study of their composition. While the quality and authenticity studies are carried out using infrared spectroscopy [17, 18], sensory analysis [19, 20] and the electronic nose [21, 22], mainly to differentiate between different varieties and brands.

Electronic noses due to its many advantages, are increasingly used to quickly assess the quality and authenticity of some food products. The electronic nose is an analytical instrument designed to rapidly detect and distinguish mixtures of aromatic substances [23]. In the research described in this paper there is used electronic nose based on ultra-fast gas chromatography (FAST-GC). This type of electronic nose is a combination of features of FAST-GC with the advantages of an e-nose based on sensors. As a result of one analysis, it is possible to obtain information concerning the composition of the tested samples and their volatile fraction profile, which give a complete information about the similarity of the sample to the reference sample [24]. It will be checked if it is possible to quickly differentiate vodka and whisky from produced in the home condition moonshine by using e-nose based of ultra-fast gas chromatography.

2. Experimental

2.1 Samples, sample preparation

The investigation was conducted on 12 samples of different spirits beverages including: Tennessee Whiskey, moonshine produced from grains including corn, two bourbons, five Scotch whisky, moonshine unknown botanical origin, vodka produced by folk recipe and the same brand of vodka produced by moonshine recipe.

All samples were prepared using the same analytical procedure. It is caused mainly by a comparable content of ethanol in the samples. Volume of 6.25 ml of deionized water and 1.75 ml of alcohol were added into 20 ml vial then incubated for 20 minutes at 40 °C. Samples were injected by the use of gas syringe held at a temperature of 100 °C.

2.2 Electronic nose analysis

In this study electronic nose Heracles II based on FAST-GC (Alpha MOS, Toulouse, France) was used. Heracles II is equipped with the injector, sorption trap, that

allows introduction of gas and liquid samples, two parallel connected chromatographic columns with different polarity of stationary phase (MXT-5 non-polar and MXT-1701 semi-polar), two flame ionization detectors (FID), dedicated software AlphaSoft V12 with implemented modules for chromatographic, chemometric and sensory analysis, Arochembase library V4HERACLES V12, dedicated autosampler HS100. The volume of 2.5 ml of the sample was introduced in 15 seconds to the injector at a temperature 200 °C at a volumetric flow rate of the carrier gas equal to 30 ml/min. The sample was hold in the sorptive trap for 20 s at 40 °C. The analysis began at 40 °C for 2 s then uses the temperature increase of 3 °C/s until reaching a temperature of 270 °C which was held for 18 seconds. As the carrier gas, hydrogen was used. For analysis of the data were used Principal Component Analysis (PCA), Discriminant Function Analysis (DFA), Soft Independent Modelling of Class Analogies (SIMCA) and Statistical Quality Control (SQC).

3. Results and discussion

The PCA method is used to reduce the dimensionality of the dataset. It allowed for the distinction of all study groups. Points belonging to groups 1, 2 and 3 are located on the left side of the PCA plane (Fig. 1). The corn was the raw material for the production of this type of alcohol samples. Points belonging to groups 4, 5, 6 and 7 are on the right side of the PCA plane. Alcohols associated to these point of groups are not produced from corn. Points belonging to the group 5 are farthest in relation to the other groups on the PCA plane. Points belonging to groups 6 and 7 are located closest from other ones in the PCA plane. This is connected with that the both groups are the representation of vodka samples produced by the same manufacturer, but by the use of different methods.

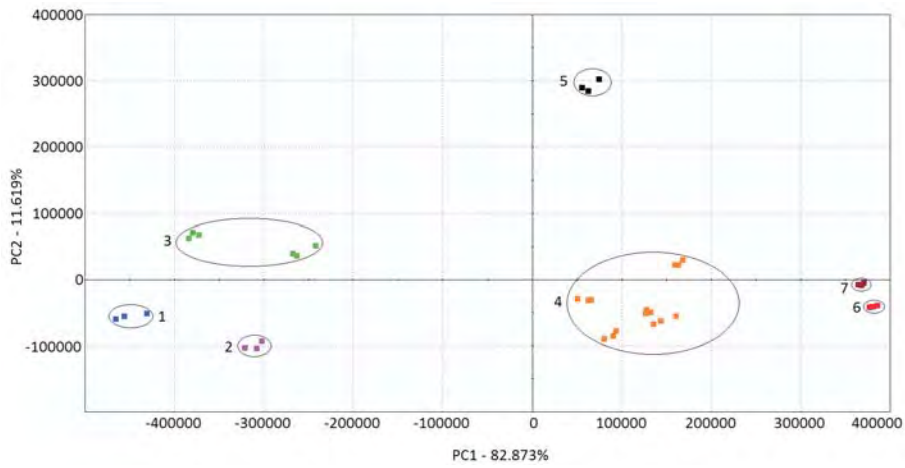


Fig. 1. Diagram showing the PCA analysis: group 1 – Jack Daniels, group 2 – moonshine no. 1, group 3 – bourbons, group 4 – whisky, group 5 – moonshine no. 2, group 6 – vodka, group 7 – moonshine vodka.

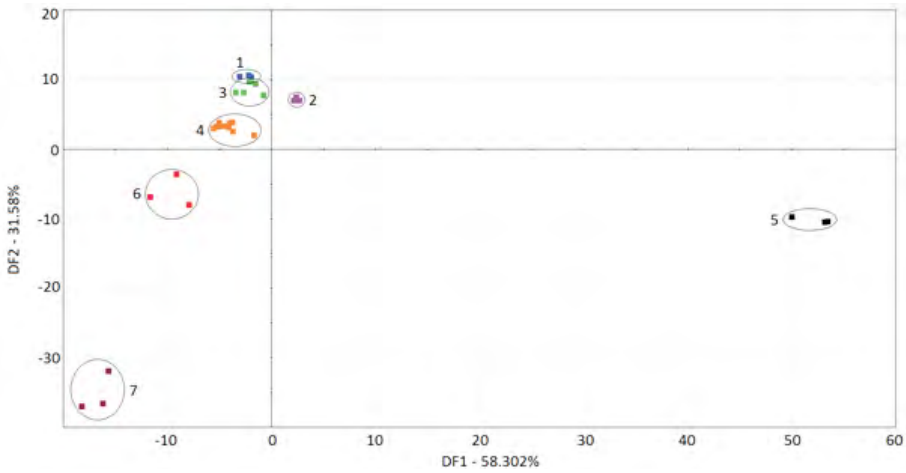


Fig. 2. Diagram showing the DFA analysis: group 1 – Jack Daniels, group 2 – moonshine no. 1, group 3 – bourbons, group 4 – whisky, group 5 – moonshine no. 2, group 6 – vodka, group 7 – moonshine vodka.

The DFA analysis made it possible to distinguish majority of the groups. Distance between groups of points 1 and 3 are too small to make proper differentiation whether they were distinguished on the DFA plane (Fig. 2). Near to points of the group 1 and 3 are located 2 and 4 group of points. The points included in groups 1, 2, 3 are the representation of alcohols produced partially from the corn distillate. Therefore these points are in close range to each other on the DFA plane. The group 4 includes whisky, which are produced by similar procedure as the other 3 groups neighbor located. Points belonging to the group 5 are located furthest from the other points on the DFA plane. This fact indicates the large differences in the composition of samples belonging to this group of samples from the other ones, it can be caused by different origin of raw materials and by various production method.

The SIMCA analysis made it possible to distinguish between all studied groups of alcohols (Fig. 3). In this case, the reference group was a group of points labeled with number 4. All the points belonging to the reference group are located in a designated area, i.e. "the envelope of confidence". Points belonging to the other groups are far away from the reference group on the SIMCA plane. In the SIMCA graphs made for other groups of points as reference groups, all points belonging to the reference groups were in "the envelope of confidence". In all of the SIMCA graphs, the points belonging to the other groups than reference groups were outside of the designated area.

The SQC analysis allowed for discrimination of all studied groups of alcohols. This type of data analysis is used to check the quality of the samples. On Fig. 4 and 5 SQC graphs are shown for which the reference groups are respectively 4 and 6 group of points. Analysis of SQC made for the other group of points, also allowed to differentiate the reference groups.

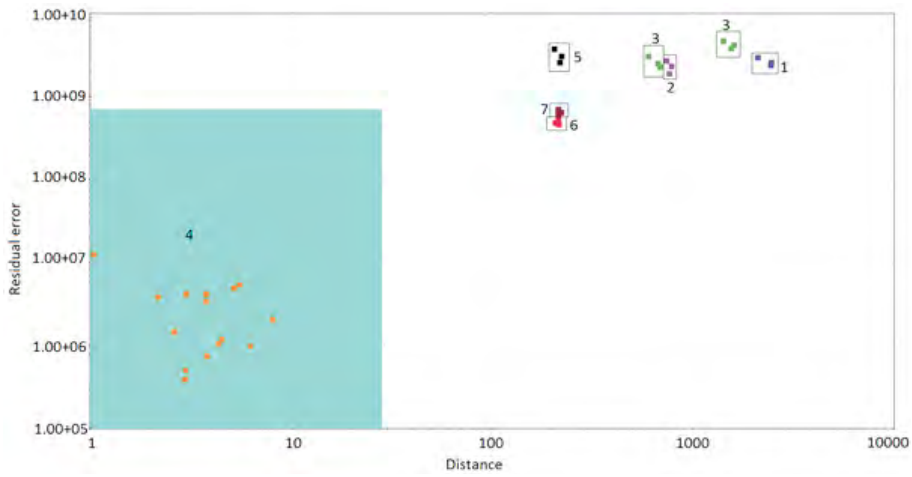


Fig. 3. Diagram showing the SIMCA analysis: group 1 – Jack Daniels, group 2 – moonshine no. 1, group 3 – bourbons, group 4 – whisky, group 5 – moonshine no. 2, group 6 – vodka, group 7 – moonshine vodka.

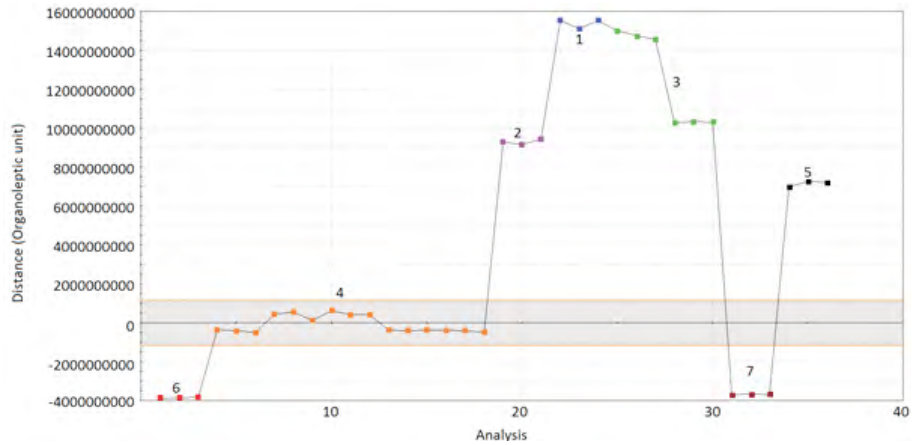


Fig. 4. Diagram showing the SQC analysis: group 1 – Jack Daniels, group 2 – moonshine no. 1, group 3 – bourbons, group 4 – whisky, group 5 – moonshine no. 2, group 6 – vodka, group 7 – moonshine vodka.

4. Conclusion

The PCA, SIMCA and SQC analysis allowed to the distinction between all studied groups of alcohols. The DFA analysis did not allow to distinguish from each other of Jack Daniels and bourbons. This is probably related to the similarity of the composition of the samples. Points belonging to moonshine no. 2 were farthest from other points on the PCA and DFA graphs. Points belonging to the moonshine

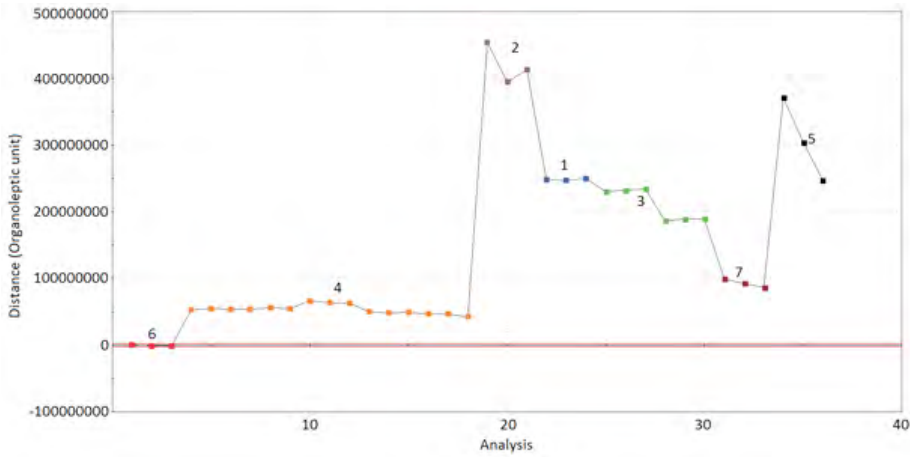


Fig. 5. Diagram showing the SQC analysis: group 1 – Jack Daniels, group 2 – moonshine no. 1, group 3 – bourbons, group 4 – whisky, group 5 – moonshine no. 2, group 6 – vodka, group 7 – moonshine vodka.

no. 1 were close to points belonging to alcohols produced from corn distillates on the PCA and DFA planes. Despite the similarity to whisky and vodkas, both moonshine were distinguished from samples of vodka, whisky and bourbons. It can be concluded that the e-nose based on FAST-GC equipped with chemometric methods such as PCA, DFA, SIMCA and SQC is an effective tool for rapid distinguishing alcohol products due to their botanical origin among the various groups of spirits which are whisky, bourbons, moonshine and vodka.

Acknowledgments

The authors acknowledge the financial support for this study by the Grant No. 2012/05/B/ST4/01984 from National Science Centre of Poland.

References

[1] Regulation (EC) No 110/2008 of the European Parliament and of the Council of 15 January 2008 on the definition, description, presentation, labelling and the protection of geographical indications of spirit drinks.
[2] Act of 13 September 2002 on the spirit drinks, No 1362/166 J. Laws, 10582–10589.
[3] Christoph N., Bauer-Christoph C.: Vodka. In: *Flavours and Fragrances Chemistry, Bioprocessing and Sustainability*. Berger R.G. (edit.) Berlin, Springer 2006, p. 231.
[4] Rhodes C.N., Heaton K., Goodall I., Brereton P.A.: *Food Chem.* **114** (2009), 697–701.
[5] Boles E.B.: *Illegal Odyssey: 200 Years of Kentucky Moonshine.* AuthorHouse 2003.
[6] Popova S., Rehm J., Patra J., Zatonski W.: *Alcohol and alcoholism* **42** (2007), 465–473.
[7] http://bc.ias.org.uk/pdfs/project-meeting-stockholm/apn-amphora-unrecorded-alcohol.pdf (accessed: 17th April, 2015)
[8] Pereira E.V.S., Oliveira S.P.A., Nóbrega I.C.C., Lachenmeier D.W., Araújo A.C.P., Telles D.L., Silva M.: *Quím. Nova* **36** (2013), 822–825.
[9] Lachenmeier D.W., Anh P.T.H., Popova S., Rehm J.: *Int. J. Environ. Res. Public Health* **6** (2009), 2090–2101.

[10] Siříšťová L., Přinosilová Š., Riddellová K., Hajšlová J., Melzoch K.: *Czech J. Food Sci.* **30** (2012), 474–482.
[11] Ng L.-K.: *Anal. Chim. Acta* **465** (2002), 309–318.
[12] Barbeira P.J.S., Stradiotto N.R.: *Fresen. J. Anal. Chem.* **361** (1988), 507–509.
[13] Marti M.P., Boque R., Busto O., Guasch J.: *Trends Anal. Chem.* **24** (2005), 57–66.
[14] Garcia J.S., Vaz B.G., Corilo Y.E., Ramires C.F., Saraiva S.A., Sanvido G.B., Schmidt E.M., Maia D.R.J., Cosso R.G., Zacca J.J., Eberlin M.N.: *Food Res. Int.* **51** (2013), 98–106.
[15] Mignani A.G., Ciaccheri L., Gordillo B., Mencaglia A.A., González-Miret M.L., Heredia F.J., Culshaw B.: *Sens. Actuators B* **171** (2012), 458–462.
[16] Cardeal Z.L., Marriott P.J.: *Food Chem.* **112** (2009), 747–755.
[17] Sujka K., Koczoń P.: *Zeszyty Problemowe Postępów Nauk Rolniczych* **571** (2012), 107–114.
[18] Adam T., Duthie E., Feldmann J.: *J. Inst. Brew.* **108** (2002), 459–464.
[19] Sujka K., Koczoń P., Górska A., Wirkowska M., Reder M.: *Żywność. Nauka. Technologia. Jakość* **4** (2013), 184–194.
[20] Parker I.G., Kelly S.D., Sharman M., Dennis M.J., HowieD.: *Food Chem.* **63** (1998), 423–428.
[21] Ragazzo-Sanchez J.A., Chalier P., Chevalier D., Ghommidh C.: *Sens. Actuators B* **114** (2006), 665–673.
[22] Lee K.Y.M., Paterson A., Piggott J.R., Richardson G.D.: *Food Qual. Prefer.* **12** (2001), 109–117.
[23] Dymerski T.M., Chmiel T.M., Wardencki W.: *Rev. Sci. Instrum.* **82** (2010), 111101–111132
[24] http://www.alpha-mos.com/analytical-instruments/heracles-electronic-nose.php (accessed: 17th April, 2015)

Determination of 3,4-methylenedioxy-α--pyrrolidinobutiophenone (MDPBP) in blood. A case report.

Marek Wiergowski[a], Mateusz Woźniak[b,*], Marzena Kata[a], Marek Biziuk[b]

[a] *Medical University of Gdańsk, Faculty of Medicine, Chair & Department of Forensic Medicine, 23 Dębowa Str., 80-204 Gdańsk, Poland*
[b] *Gdańsk University of Technology, Chemical Faculty, Department of Analytical Chemistry, 11/12 Narutowicza Str., 80-233 Gdańsk., Poland* ✉ *womat90@gmail.com*

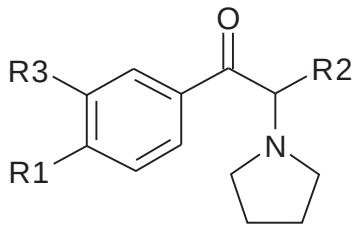
Keywords
blood
cathinones
drug abuse
GC-MS toxicological screening
MDPBP

Abstract
Synthetic drugs, known as "legal highs", are still very popular on black market. The determination of their composition are required in courts of law and also to understand what potential users may be subjected to. The interest of new cathinones, e.g. MDPBP, is in view of psychoactive effects that they cause and that, in many cases, they are not controlled by law. The case outlined here concerns 19 years-old man, who was found dead with an envelope containing white powder laying nearby the cadaver. The analyses of the powder, revealed the presence of MDPBP. Blood was investigated for routine toxicological analysis for traditional drugs and for MDPBP by liquid-liquid extraction procedure with derivatization followed by GC/MS analysis. The results showed very high concentration of MDPBP, what probably was the reason of death. To our knowledge, this was the second fatal accident confirmation of MDPBP abuse in the Poland.

1. Introduction

Recently, it has been a significant rise in the popularity of "legal highs", especially among young people. A novel group of these drugs classified to cathinones, contain α-pyrrolidinophenones fragment, were emerged on black market in last decade (Fig. 1). Most synthetic cathinones originate in China and, to a lesser extent, in India and in Europe [1, 2].

MDPBP, (*RS*)-1-(3,4-methylenedioxyphenyl)-2-(1-pyrrolidinyl)-1-(butanone), is one of the new drugs available for sale since 2009 (e.g. via internet). Usually it is mixed with flephedrone, pentedrone, pentylone, MAPP and MDPV. Its presence was confirmed in legal highs, such as: White Fiz, Vanilla Sky, and in Bath Salts. Doses which affect central nervous system and are usually ingestion range from 50 to 100 mg. MDPBP is the most commonly administered via oral ingestion, nasal insufflations, smoking or intravenous injections. The pharmacokinetics and psychoactive effects have not been fully-known yet, but it is believed that they are



Compound	R1	R2	R3
PPP	–H	$-CH_3$	–H
MPPP	$-CH_3$	$-CH_3$	–H
Pyrovaleron	$-CH_3$	$-C_3H_7$	–H
MPBP	$-CH_3$	$-C_2H_5$	–H
PVP	–H	$-C_3H_7$	–H
MDPPP	$-O-CH_2-O-$ (R1–R3)	$-CH_3$	–
MDPBP	$-O-CH_2-O-$ (R1–R3)	$-C_2H_5$	–
MDPV	$-O-CH_2-O-$ (R1–R3)	$-C_3H_7$	–

Fig. 1. Structures of some α-pyrrolidinophenones [1].

similar to amphetamine, cocaine and to other substances containing α-pyrrolidinophenones fragment. Its use has been reported to cause states of euphoria, agitation, hallucinations, and aggressive behavior. Overdoses cause confusion, acute poisoning, increase heart rate, high blood pressure and finally could lead to arrhythmia, myocardial ischemia, and death [3, 4].

The case described herein concerns a 19 years-old man who was found naked dead on the field. Small bag with white powder was also found nearby. It was believed, that it was ingested by man before death. However, his friends testified that deceased was on the party a day before accident, but he unexpectedly fled. They also reported, that man drank only approximately 100 mL of vodka and that drugs were not used during the party. Moreover, the deceased was not agitated or aggressive. Additional testimony of his father indicates that subject did not have a habit of taking drugs or alcohol.

The preliminary toxicological analysis of the powder by various of mass spectrometry methods revealed the presence of MDPBP as the only active compound in the powder. Due to this, the aim of this study was to determinate MDPBP in blood obtained during autopsy. However, abusers often ingest more than one drug. Due to this, blood sample was also investigated for psychoactive classical drugs, amphetamine, methamphetamine and their derivatives, cannabinoids, cocaine and its metabolites by ELISA immunoenzymatic test (Neogen). For confirmation presence of cannabinoids and quantitative determination

of Δ^9-tetrahydrocannabinol (Δ^9-THC) and its main metabolite 11-nor-Δ^9-tetrahydrocanabinol-9-carboxylic acid (THC-COOH) gas chromatography coupled with MS detector was also performed.

2. Experimental

2.1 Chemicals

Methanolic solutions of 3,4-methylenedioxy-α-pyrrolidinobutiophenone hydrochloride (MDPBP.HCl) at concentration 1.0 mg mL^{-1} and methamphetamine-D5 (mAMP-D5) at concentration 0.1 mg mL^{-1} were used as standards and were obtained from LGC Standards (London, United Kingdom).

All solvents used were of HPLC grade purity and were obtained from Sigma Aldrich. Anhydride trifluoroacetic anhydride (TFAA) and 1-chlorobutane were ≥99 % purity (analytical grade) and were also purchased from Sigma Aldrich. Hydrochloric acid at concentration 35–38 %, potassium carbonate and sodium chloride were of analytical grade and were obtained from POCH (Gliwice, Poland). To obtain 5 mol dm^{-3} K_2CO_3, 691 g of this salt in powder was placed in 1 L volumetric flask and filled up to mark with water. Saturated NaCl solution was prepared by dissolving 35.9 g of NaCl in powder with 100 mL of water.

2.2 Preparation of standard and calibration solutions

Original MDPBP.HCl and mMAP-D5 standard solutions were used as a stock solution and the internal standard (IS) respectively.

The calibration solutions were prepared by dissolving stock solution with methanol (working solution). Subsequently, appropriate amount of the working solution was mixed with drugs-free blood and whole extraction procedure was performed. The amount of IS added to each calibration solution was 1 μg. Calibration curve was generated in the range from 50 to 500 ng mL^{-1} by plotting the ratio of the peak areas of MDPBP.HCl/IS-mAMP-D5 against the concentration of MDPBP.HCl.

2.3 Samples, sample preparation

The blood samples were obtained during autopsy and were stored at +4 °C before the analysis. 1 mL of blood was used for analysis.

Sample preparation was performed using modified method according to in-house prepared method for the determination of amphetamines and piperaznes in blood and urine by liquid-liquid extraction procedure with derivatization followed by GC/MS analysis. Blood (1 mL), solution of IS (10 μL), K_2CO_3 at concentration 5 mol dm^{-3} (2 mL) and saturated NaCl (2 mL) and acetonitrile (2 mL) were added to the screw-capped glass centrifuge tube. The tube was

vortex-mixed for 1 min. Subsequently, 1-chlorobutane (2 mL) were added and sample was vortex-mixed for 2 min. The solution was centrifuged for 3 min at 3000 rpm. The top layer was then transferred to new glass tube. An additional 2 mL of 1-chlorobutane were added to the blood after first extraction, the tube was vortexed for 2 min and then centrifuged for 3 min at speed 3000 rpm. The top layer was added to tube containing the first extracts. The bottom layer was discarded. Then 100 μL of hydrochloride acid solution in methanol (1:9, v/v) was added. Then, extracts were evaporated to dryness with an inert gas stream (N_2) at 40 °C and reconstituted in 50 μL ethyl acetate. Derivatization was performed with 50 μL TFAA (20 min, 55 °C). Finally, solution was evaporated to dryness and residue was dissolved in 50 μL of ethyl acetate. An aliquot of 2 μL volume was injected into the GC-MS system.

2.5 Instrumentals

The chromatographic separation was carried out using an GC-7890A gas chromatograph (Agilent Technologies) equipped with autosampler G4567A (Agilent Technologies), split/splitless injector and mass spectrometry detector MS-5975C (Agilent Technologies). The analytes were separated on capillary column ZB-5 MS 30 m × 0.25 mm i.d., 0.25 μm film thickness (Phenomenex) with helium as carrier gas (1.0 mL min^{-1}). Split injection mode was used (10:1). The oven temperature was programmed as follows: 1 min at 50 °C, then 30 °C/min up to 160 °C and finally, 5 °C/min up to 250 °C hold for 1 minute. Inlet temperature was 300 °C, detector mass spectrometry source ions temperature was 280 °C and the MS quadrupole analyzer temperature was 200 °C. For full-scan acquisition the MS was operated in positive electron-impact mode (electron energy 70 eV) and the mass detection range was *m/z* 30–380. For selected ion monitoring (SIM), 112 and 46 ions (quantitative confirmation) and 113, 70 and 114, 159 ions (qualitative confirmation), respectively for MDPBP and IS, were chosen on the basis of their abundance and *m/z* values.

3. Results and Discussion

The chemical and toxicological study of blood samples showed the presence of psychoactive MDPBP and cannabinoids in the psychoactive form (9-THC) and its inactive main metabolite of THC-COOH in the concentrations showed in Table 1.

High level of MDPBP determined in blood confirmed ingestion of this drug before death. Probably, it was the main reason of the death. The presence of small amount of metabolite of cannabinoids proves early hashish or marijuana use with no significant effect of these toxic substances and may indicates the tendency for drug use by this man. Fatal cases caused by overdose of cannabinoids are very rare and often are associated with taking other psychoactive substances at the same time.

Table 1
Concentration of drugs in blood obtained during toxicological analysis.

Analyte	Concentration [ng mL^{-1}]
MDPBP	9.322
Δ^9-THC	Below *LOQ* (<1)
THC-COOH	6

To our knowledge, this was the second fatal accident confirmation of MDPBP abuse in the Poland. The first one concerned 19 years-old man who died after overdose of drugs [4]. Toxicological examination showed that a concentration of MDPBP in his blood was 7.010 ng mL^{-1}, what is comparable to results obtained in our case (9.322 ng mL^{-1}) [4]. These authors also reported a case of four drivers controlled by the Police, because of suspicious of driving under the influence of drugs. Blood analysis of these drivers showed the concentration of MPDBP in the range from 22 to 92 ng mL^{-1}. Above-mentioned fatal concentrations of MDPBP were about 70–90-times higher than values determined in blood of drivers suspected of driving under the influence of drugs. Another confirmation case of MDPBP abuse was reported in Czech Republic in 2013 [6]. In blood of 36 years-old man, who was treated for his long-term ethanol addiction and a habit of experimenting with new drugs available in the local black market shops, was determined MDPBP and one other substance, which was recognized as a metabolite of this drug. Patient admitted to use an illegal powdered drug that was distributed under the name "Funky", to overcome the symptoms of ethanol withdrawal.

Calibration curve was linear in the studied concentration range (50–500 ng mL^{-1}) with correlation coefficients of 0.9854. The weighing factor of $1/x$ was applied to calibration curve in order to increase the accuracy in the lower concentration range. The limit of detection (*LOD*) was 34.8 ng mL^{-1} based on following formula

$$LOD = 3.3 \times (s_b/a) \quad (1)$$

where s_b is the standard deviation of the intercept and a is the slope of the calibration curve. The limit of quantification (*LOQ*) was 104 ng mL^{-1}, calculated as three times *LOD* value [5].

4. Conclusions

Blood is one of the most commonly used matrixes in forensic toxicology analysis for drug or other toxic substances screening. To confirm the presence of xenobiotics or their metabolites, urine may be also analysed. However, due to the

rapidly growing numbers of new psychoactive substances available on black market and the large variability of drugs in structure and in concentration, the forensic investigation of intoxications or fatalities is relatively difficult. Therefore, new methods for the analysis of novel drugs are required.

Proposed method for the determination of MDPBP in blood, seems to be sufficiently sensitive for identification fatal cases with relatively high concentration of MDPBP in blood, when high dose of drug was taken (high value of *LOD* and *LOQ*). Therefore, more sensitive and selective gas or liquid chromatography method coupled with tandem mass spectrometry is recommended in non-fatal cases of MDPBP poisoning.

References

[1] Westphal F., Junge T., Klein B., Fritschi G., Girreser U.: *Forensic Sci. Int.* **209** (2011), 126–132.
[2] Spiller H.A., Ryan M.L., Weston R.G., Jansen J.: *Clin. Toxicol.* **49** (2011), 499–505.
[3] Zawilska J.B., Wojcieszak J.: *Forensic Sci. Int.* **231** (2013), 42–53.
[4] Gieroń J., Adamowicz P.: *Materiały XXXI Konferencji Toksykologów Sądowych*. Ciechocinek (2014), 55–56.
[5] Konieczka P., Namieśnik J.: Walidacja procedur analitycznych. In: *Ocena i kontrola jakości wyników pomiarów analitycznych.* Konieczka P., Namieśnik J. (edits.). Warszawa, WNT 2007, p. 225–299.
[6] Balikova M., Zidkova M., Oktabec Z., Maresova V., Linhart I., Himl M., Novotny M.: *J. Forensic. Toxicol. Pharmacol.* **2** (2013), 1–4.

Development of the poly(methylene blue) modified graphite electrodes for the electrochemical detection of hydrogen sulphide

MAGDA ZLÁMALOVÁ[a, b, *], PAVEL JANDA[a], KAREL NESMĚRÁK[b]

[a] *Department of Electrochemical Materials, J. Heyrovský Institute of Physical Chemistry of the ASCR, v. v. i., Dolejškova 2155/3, 182 23 Prague 8, Czech Republic* ✉ *magda.zlamalova@jh-inst.cas.cz*
[b] *Department of Analytical Chemistry, Charles University in Prague, Hlavova 2030/8, 128 43 Prague 2, Czech Republic*

Keywords
cyclic voltammetry
electropolymerization
hydrogen sulphide
poly(methylene blue)
potentiometry

Abstract
A comparative study have been undertaken of the electropolymerization and electrochemical properties of deposited electroactive polymer on two different graphite electrode substrates: basal plane highly oriented pyrolytic graphite (HOPG) and pencil-graphite electrode (PGE). Poly(methylene blue) modified electrodes (HOPG/pMB and PGE/pMB) have been prepared by potential cycling in aqueous electrolyte solution containing methylene blue monomer. It has been found that electrochemical properties and electrocatalytic activity of deposited film are greatly influenced by the type of electrode substrate. Developed electrodes have been further investigated as potential sensors for hydrogen sulphide.

1. Introduction

Controlled modification of the electrode surface can produce electrode systems with new interesting properties, such as mediation or catalysis of new charge transfer reactions, protection of the surface against passivation or even change of the electrochemical reaction mechanism. This all can lead to improvement of electrochemical response to certain analytes. Utilizing electrodes with immobilized suitable chemical mediators can solve certain problems with bare solid electrodes such as insufficient sensitivity and selectivity, slow electron transfer, high overpotential necessity, current or potential oscillations.

Over the past two decades polymer modified electrodes have been extensively studied due to their application in electrochromic devices, electrocatalysis and electroanalysis [1, 2]. Electrochemical polymerization is one of the most common and useful techniques to obtain polymer thin film onto electrode surface. Compared to the chemical preparation of polymeric films it has some advantages,

such as high reproducibility, thickness control and simple preparation methodology. In the past few years, surface-modified electrodes based on the electropolymerization of various phenoxazine and phenothiazine derivatives have been reported in the literature [3, 4].

Methylene blue (MB), a phenothiazine derivative, is a water-soluble cationic dye. Potential cycling in aqueous solution containing MB monomer leads to the formation of stable conductive polymer film on the solid electrode surface. It has been already published that basic solutions are optimal media for the polymerization of MB [5]. The electrocatalytic activity of poly(methylene blue) (pMB) in presence of some biologically active compounds has been already reported in several studies [5–9]. Thanks to the strong adhesion of the polymer to the solid substrate surfaces pMB films exhibit long-term mechanical stability.

The aim of our work is designing new electrode system which combines auspicious electrochemical properties of pMB and advantages of HOPG and PGE. Our findings will be utilized for development of new potentiometric sensor.

2. Experimental

2.1 Reagents and chemicals

Methylene blue was purchased from Lachema and used as received. The supporting electrolyte solution used for the electropolymerization of MB consisted of 0.1 M phosphate buffer and 0.1 M sodium nitrate (pH = 8.0). The concentration of monomeric MB dissolved in the solution was 0.1 mM. Solution of 0.1 M phosphate buffer with 0.1 M KCl was used for pMB electrochemical characterization and for other experiments. Solutions of sodium sulphide were prepared immediately before experiments. Millipore Milli-Q pure water (resistivity ≥ 18 MΩ cm) and analytical grade reagents were used for preparation of all solutions. Stock solutions were kept in glass vessels in dark at ~6 °C. All experiments were performed at room temperature.

2.2 Instrumentation

All electrochemical experiments were carried out on computer-controlled potentiostat-galvanostat Wenking POS2 (Bank Electronik) with software CPC-DA (Bank Elektronik) in a three-electrode electrochemical cell of volume 2 ml with bare or modified HOPG or PGE as working electrodes, saturated calomel reference electrode (SCE), and platinum wire counter electrode. The pH measurements were carried out with a pH-meter (Jenway 3510) at room temperature. Nanomorphology of pMB layer was examined by the atomic force microscope (NanoScope IIIa Multimode, Bruker) in the semicontact mode to minimize tip/surface interactions. The Si cantilevers (OTESPA) were oscillated at ~300 kHz.

2.3 Preparation of pMB-modified HOPG and PGE

Graphite substrate was prepared by peeling off basal plane of highly oriented pyrolytic graphite (HOPG, ZYB Grade, 12×12× 2 mm; Momentive Performance Materials Quartz). Geometric area accessible for modification was defined by viton flat sealing (20 mm^2). The pencil-graphite rod (KOH-I-NOOR, HB of 0.5 mm in diameter and 6 cm in length) was inserted into a glass capillary and sealed using two-component epoxy adhesive. Electrical contact was obtained by mercury with platinum wire. The electrochemical polymerization of methylene blue was carried out under argon atmosphere in an one-compartment electrochemical cell containing methylene blue monomer solutions in 0.1 M phosphate buffer (pH = = 8.0) with 0.1 M $NaNO_3$ saturated by argon. The preparation of pMB films was carried out by voltammetric cycles between -0.6 V and +1.1 V at the sweep rate 100 mVs^{-1}. The polymer growth was controlled by the deposition time or the number of deposition cycles. After electropolymerization both the electrode and the cell were thoroughly rinsed with phosphate buffer containing 0.1 M KCl to remove any remaining monomeric MB.

3. Results and discussion

Fig. 1 shows typical cyclic voltammograms obtained during the film growth. The voltammetric curves are similar to that obtained on glassy carbon electrode [5]. Beyond cca 0.8 V the rapidly rising current (wave III) originates from the oxidation of the MB to a cation-radical $MB^{+\cdot}$ formed by the polymerization of monomer. The

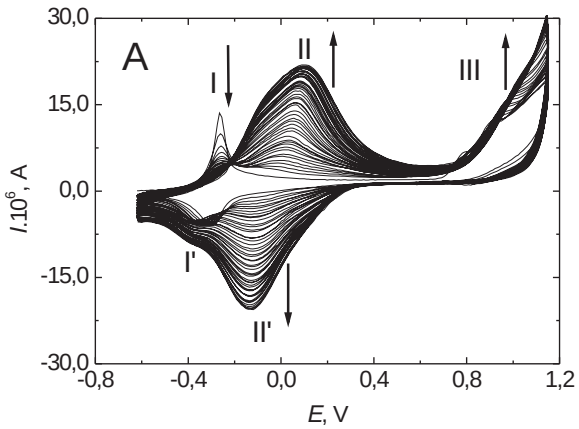


Fig. 1. The pMB film growth during electropolymerization from a solution containing 0.1 mM MB monomer in 0.1 M phosphate buffer and 0.1 M $NaNO_3$ (pH = 8.0) on the surface of HOPG (A) and PGE (B). 30 minutes cycling between −0.6 and +1.1 V *vs.* SCE at scan rate 100 mV s^{-1}.

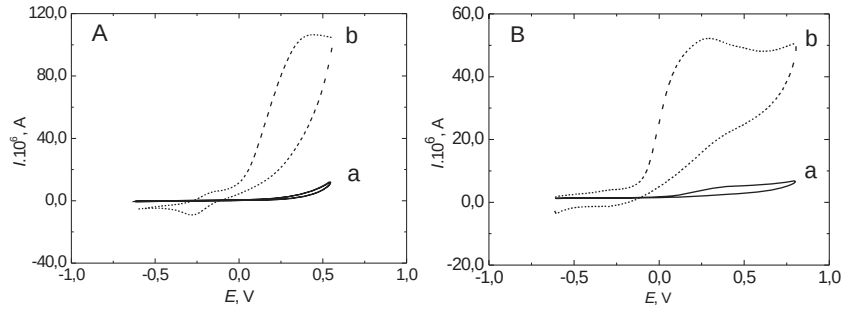


Fig. 2. The cyclic voltammetry curves of HOPG/pMB (A) and PGE/pMB (B) in 0.1M phosphate buffer with 0.1M KCl (pH = 8.0) containing 0.01M Na_2S. Curves a) on bare electrodes and curves b) on pMB modified electrodes.

growth of the polymer film is accompanied by a decrease of the monomer oxidation peak at –0.26 V (I, I') and by an increase of redox waves corresponding to the polymeric MB (II, II'). We observed that the oxidation peak I is decreases in subsequent cycles. It was suggested that initial increase of peak current is in consequence of MB adsorption on the graphite surface. Adsorbed MB monomer shows an oxidation peak at the same potential as that of the monomer from solution.

Cyclic voltammetry was performed to investigate catalytic activity of HOPG/pMB towards sodium sulphide as a model HS^-/S^{2-} analyte. For this measurement sodium sulfide was dissolved in 0.1 M phosphate buffer with 0.1 M KCl (pH = 8.0). With the first dissociation constant of hydrogen sulfide in aqueous solutions pK_{a1}~7.02 all hydrogen sulphide is practically in the form of HS^-. The cyclic voltammetry behaviour in the absence and presence of 0.01M Na_2S in 0.1M phosphate buffer and 0.1M KCl (pH = 8) at the bare HOPG and the HOPG/pMB is shown in Fig. 2 A. New oxidation peak at potential about 0.340 V was observed. No oxidation peaks corresponding to the sulfhydryl anion oxidation were observed for the bare HOPG. In the case of PGE, after modification by pMB the oxidation peak significantly increased. Since PGE surface area is a hundred times lower than HOPG surface, the current responses were converted on the current density. This recalculation showed that the electrocatalytic activity of PGE/pMB to sulfhydryl group greatly exceeds electrocatalytic activity of HOPG/pMB (Fig.3).

Atomic force microscopy (AFM) images of the HOPG/pMB provided detailed information about changes in the surface nanomorphology and homogeneity of the deposited film. After modification, spherical nanostructures were found on HOPG basal planes forming relatively homogeneous layer. No significant changes in the nanomorphology of pMB film were found after voltammetric measurement in presence of sulfides.

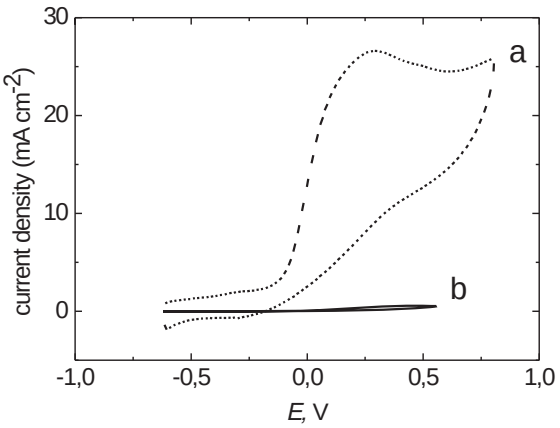


Fig. 3. Cyclic voltammograms of PGE/pMB (a) and HOPG/pMB (b) in phosphate buffer with 0.1M KCl (pH = 8.0) containing 0.01M Na_2S after recalculation towards surface area of used electrodes.

4. Conclusions

We have investigated electrochemical behavior during electropolymerization and electrochemical properties of resulting HOPG/pMB and PGE/pMB electrodes. Based on cyclic voltammetry measurements, we can conclude that both modified electrodes exhibit electrocatalytic activity towards sulfhydryl group. Deposited pMB film on PGE substrate greatly exceeds electrocatalytic activity of the film on basal plane HOPG. Investigated pMB-modified pencil graphite electrode can be utilized for the construction of new potentiometric sensor.

Acknowledgments

This work was supported by grant project SVV260084 of Charles University in Prague.

References

[1] Carpi F., De Rossi D.: *Opt. Laser Technol.* **38** (2006), 292–305.
[2] Fungo F., Jenkhe S.A., Bard A.J.: *Chem. Mater.* **15** (2003), 1264–1272.
[3] Schelreth D.D., Karyakin A.A.: *J. Electroanal. Chem.* **395** (1995), 221–232.
[4] Barsan M.M., Pinto E.M., Brett C.M.A.: *Electrochim. Acta* **53** (2008), 3973–3982.
[5] Karyakin A.A., Strakhova A.K., Karyakina E.E., Varfolomeyev S.D.: *Bioelectrochem. Bioenerg.* **32** (1993), 35–43.
[6] Marinho M.J.C, Cabral M.F., Mazo L.H.: *J. Electroanal. Chem.* **685** (2012), 8–14.
[7] Komura T., Niu G.Y., Yamaguchi T., Asano M., Matsuda A.: *Electroanalysis* **16** (2004), 1791–1800.
[8] Silber A., Hampp N., Schuhmann W.: *Biosens. Bioelectron.* **11** (1996), 215–223.
[9] Rincón R.A., Artyushkova K., Mojica M., Germain M.N., Minteer S.D., Atanassov P.: *Electroanalysis* **22** (2010), 799–806.
[10] Liu J., Mu S.: *Synthetic Metals* **107** (1999), 159–165.

Indexes

Author Index

Keyword Index

Proceedings of the 11th International Students Conference "Modern Analytical Chemistry"

Edited by Karel Nesměrák.
Published by Charles University in Prague, Faculty of Science.
Prague 2015.
1st edition – viii, 242 pages

ISBN 978-80-7444-036-6

ISBN 978-80-7444-036-6