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Prague, 22—23 September 2016

Edited by Karel Nesměrák

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Preface

The twelfth volume of the "Modern Analytical Chemistry" proceedings involves thirty seven contributions, which were presented at the conference organized by our Department of Analytical Chemistry, Faculty of Science, Charles University of Prague on 22–23 September 2016.

The mission of the conference is not only to be a forum for the presentation of achievements in the field of analytical chemistry by PhD students from various countries, but also to enhance presentation skills of the participants. Therefore the full text of contributions are published here. The reader will find that published contributions cover all branches of analytical chemistry, from improvement of instrumentation to application on environmental, heath, and toxicological problems. The contributions are assorted by the sequence of their delivering, so the detailed indexes help to reader in orientation by the name of author(s) or by keywords.

The Proceedings assure us, that analytical chemistry, mainly thanks to young analytical chemists, remains exciting and steadily developing science with new, unsuspected ways of its innovation and application. And this is what makes the organization of this meeting very fulfilling and satisfactory.

Last, but not least, we are very grateful to all sponsors, 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.

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Contents

Contributions

Determination of cyanide ion in urine samples using ion chromatography with pulsed amperometric detection

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Keywords
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IC-PAD
tobacco smoke
urine

Abstract
The aim of this research was to develop analytical procedure and its use to study the urine samples. Determined cyanide ion concentrations in forty samples of urine that were collected from healthy volunteers exposed to tobacco smoke (active smokers) and environmental tobacco smoke (passive smokers). Cyanide detection was achieved by ion chromatography with pulsed amperometric detection; limit of detection was found 0.1 µg/l.

1. Introduction

Biological materials collected from human are an excellent source of information on environmental pollution and its impact on human health and life. Cyanides are used in various areas of industrial activity, including mining and chemical industry [1]. Natural source of cyanide are cyanogenic glycosides found in the seeds of plants such as apricots, peaches, and plums [2]. Furthermore, cyanides may also be present in drinking water, soil and air in a previously mentioned industrial activities. The presence of cyanide ions in the air is the result of the issuance of car exhaust and fires. The man introduces himself cyanide into the body as a result of tobacco smoking, in addition to exposing people in his environment [3].

The toxic effects of cyanide is based on a combination of trivalent cation iron cytochrome a3, an integral component of the enzyme cytochrome oxidase located in the mitochondria of liver [4]. The combination of cyanide ion with this enzyme results in inhibition of cell respiration and severity of anaerobic glycolysis, and as a result of tissue hypoxia and metabolic acidosis. The half-life of cyanide ions in the body is about 2 hours, then they are metabolized to a less toxic form, excreted with body fluids (Fig.1) .

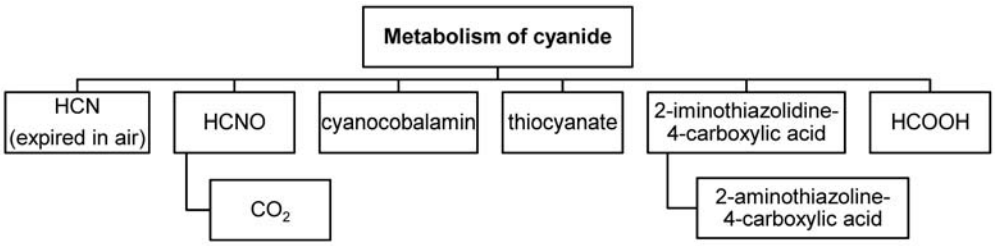

Fig. 1. Basic processes involved in the metabolism of cyanide.

It is estimated that the lethal dose for humans is 1.5 mg/kg. In view of the toxicity of cyanide, it is necessary to monitor the levels of their content in both biological and environmental materials. The recent methods cover both established and emerging analytical disciplines and include spectrophotometry, capillary electrophoresis with optical absorbance detection, atomic absorption spectrometry, electrochemical methods (potentiometry/amperometry/ion chromatography--pulsed amperometry) and gas chromatography [5]. With the development of ion chromatography as a peruse and simultaneous analytical technique, successful determination of cyanide ion in biological sample as urine has been achieved.

2. Experimental

2.1 Reagents, chemicals, and calibration solution

All reagents and chemicals (50% NaOH and NaCN) were purchased from Sigma Aldrich. Deionized water was obtained from Milipore Gradient A10 (resistivity 18.2 Ωcm at 25 °C) water purification system (Milipore, USA). To obtain stock solution of 1000mg/l NaCN, a mass of 0.0377g of this salt in powder was placed in polyethylene volumetric flask and filled up by 100 mM NaOH to a total weight of 20 g. The calibration solutions were prepared by dissolving stock solution with 100 mM NaOH.

2.2 Sample preparation

Cyanide is reactive and unstable, therefore urine samples should be stabilized as soon as it is possible by adding 100 mM NaOH to occur pH below 11. The urine sample was storage at refrigerator before analysis. A volume of 5 ml of urine was used for analysis. To remove particles sample was filtered through a Cartige II H filter (Dionex, USA). This filtrate was taken to be filtered through a 0.45μm syringe filter. The resulting filtrate was diluted three fold with deionized water and analyzed immediately. The analysis of each sample were repeated three times.

2.3 Instrumentals

Analysis was performed by ion chromatograph ICS 3000 (Dionex, USA) on anion separator column Dionex IonPac AS15 analytical guarded by anion guard column Dionex IonPac AG15. The column temperature was maintained at 30 °C, the tray temperature was set at 10 °C. The volume of sample injected was 10 µl by full loop injection. The pulsed amperometric detection was performed on certified disposable silver working electrode against pH-Ag/AgCl reference electrode in AgCl mode. The background was in the range 3–13 nC. The backpressure was at about 110 psi, and the noise was below 7 psi. The time of an analysis was 25 minutes.

3. Results and discussion

After parameter optimization, the procedure for determining cyanide ion in urine samples was validated to ensure the appropriate level of quality control and quality assurance of measurements. Calibration curve was generated in the range from 0.3 to 150 µg/l NaCN by plotting the ratio of the peak areas against the concentrations of respective standards, and expressed by the coefficient of determination (R^2 = 0.9935). The determined calibration curves were used to calculate the concentration of analytes in the urine samples. The limit of determination was set on the basis of the signal-to-noise ratio for samples of very low analyte concentration. The precision of developed procedure was expressed as the coefficient of variation calculated for three replicates. After completing the analysis with the sample without the added analytes and the sample containing added analytes,

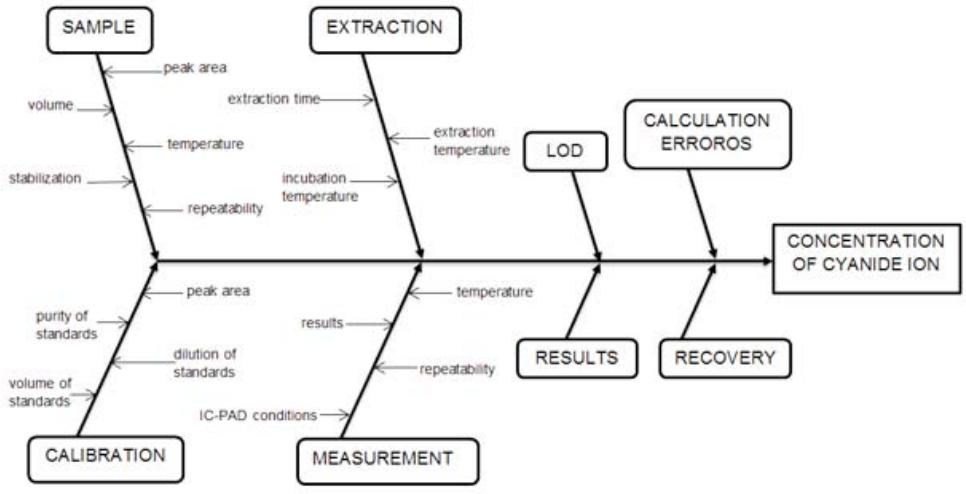

Fig. 2. The Ishikawa diagram presenting the influence of parameters in the analytical process for determination of cyanide ion in urine sample.

Table 1
Cyanide concentrations found in analysed urine samples of forty subjects.

Donor group	Active smokers	Passive smokers	Nonsmokers
Number of donors	19	7	14
Number of women	11	7	10
Average number of cigarettes smoked	6–10	–	–
Time	in the evening	–	–
Passive smoking at work	yes	yes	–
Passive smoking in the home	yes	yes	–
Average cyanide concentration [μg/l]	11.16	8.15	2.71

the recovery (101%) was calculated. The sources of uncertainty associated with the determination of cyanide detection in real urine samples are presented graphically using the Ishikawa diagram (Fig. 2). The results of linearity (y = 0.0248 x + 0.1069; R = 0,997), precision (SD:0.003, CV: 1%) and limit of detection (LOD = 0.1μg/l) are satisfying.

Urine samples were collected from volunteers smokers, passive smokers and nonsmokers. The volunteers were students Chemical Faculty of Gdańsk University of Technology. The mean concentration values of cyanide ion in urine samples were listed in Table 1. One person from the group of smokers declared occasional smoking e-cigarettes, but the analysis result was below LOD. Sample of biological material samples are difficult due to the complex matrix during the preparation of the sample for analysis, in the extraction step besides a stable complexes with metal cyanide, followed by elimination of interferes. It is necessary to add sodium hydroxide in order to stabilize the forms of occurrence of cyanide, which results in bonding of volatile forms of cyanide. None of the volunteers declared exposure to smoke from fire.

4. Conclusions

Extensive use of cyanide in the industry and their natural occurrence in plants causes a threat to the safety and human life. Compounds containing the cyanide ions are fast-acting poison, which notably disturbs the process of cell respiration, which in turn causes a number of ailments and diseases and even death. Because of the toxicity of cyanide, of particular importance is the determination of the sample materials in environmental and biological. Cyanide ions may be biomarkers of exposure to tobacco smoke components. This study proved that IC-PAD can be successfully applied for determination of cyanide ion in urine samples.

Acknowledgments

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Application of analytical procedure based on accelerated solvent extraction and ion chromatography technique for determination of thiocyanate and other inorganic ions in human placenta samples

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Keywords
biomarker
ion chromatography
placenta
thiocyanate ion
tobacco smoke

Abstract
Exposure of a pregnant woman during pregnancy is a special case of exposure to toxic substances. Samples of placenta collected for the studies had been prepared with the technique of accelerated solvent extraction and later analyzed for the presence of thiocyanate ion and other inorganic ions, with the use of the technique of ion chromatography. The concentration of thiocyanate ion in placenta samples collected from active smokers was at a higher level than that in placenta samples of non-smoking women who were not exposed to the harmful compounds of tobacco smoke in the environment. The conducted statistical of measurement data obtained the differences in the concentrations of thiocyanate ion in the marked samples. The high concentration of thiocyanate ions in the placenta samples proves that harmful substances from tobacco smoke penetrate the placenta.

1. Introduction

Tobacco smoking is a serious health problem, particularly in the case of pregnant women because there is a chance of negative effects in children exposed to tobacco smoke *in utero* [1]. A pregnant woman can be exposed to tobacco smoke components by actively smoking tobacco or being a passive smoker. A smoking woman is mainly exposed to mainstream smoke which is absorbed through her mouth during the smoke inhalation. Another exposure source is sidestream smoke which enters the environment from the lit end of the cigarette between the puffs. A female passive smoker is exposed to the components of environmental

tobacco smoke which is a mixture of sidestream smoke and exhaled mainstream smoke. Constituent of the environmental tobacco smoke diffuses into the atmosphere becoming diluted by ambient air and undergoing various physical and chemical transformations that include reactions with chemical substances not generated from burning tobacco [2, 3].

In the biomonitoring of toxic substances from tobacco to which a pregnant woman is exposed placenta samples can be used as biological material for studies, because of the fact, that it is an organ which connects two separate organisms. Placenta participates in the metabolism of chemical compounds and in their transportation to fetal bloodstream [4].

Literature information on this subject are rare and not complete information about marking nicotine and cotinine, and of selected groups of compounds [5–7] and of some metals: Fe, Cu and Zn, Cd, and Se [6, 8]. There is a lack of reports about determination thiocyanate ion in human placenta samples. Thiocyanate is the detoxification product of cyanide with a half-life of 10–14 days [9].

In this paper information on proposed analytical procedure of determination of thiocyanate and other inorganic ions in human placenta samples with the use of ion chromatography and result of analysis of real samples of placenta are presented. Samples of placenta were prepared based on accelerated solvent extraction, with water as a solvent, which significantly lowers the cost of an analysis and perfectly suits modern trends of pro-environmental analytics [10, 11].

2. Experimental

2.1 Samples collected

The placenta samples were collected from women in the department of obstetrics of the Medical University of Gdańsk, immediately after birth and placed in sterile plastic containers. They were stored in the temperature of –30 °C until the time of the analysis.

The women from whom the placenta samples were collected were asked to fill in a questionnaire concerning their habits during pregnancy. As the research was based on biological material of human origin, it required the consent of the Bioethics Committee. This was granted on 8th January, 2015 (No. NKEBN/571-/2014-2015).

2.2 Preparation of the placenta samples

Before being extracted the samples were subjected to homogenizing treatment. Then, samples of 3–5 g were prepared for accelerated solvent extraction. Model ASE 350 (Dionex), an automated extraction system for accelerated solvent extraction was used. The solvent used in the studies was water (it does not have

a harmful effect on the environment and lowers the costs of conducted studies). During the extraction the water temperature was raised to 150 °C under the pressure of 1700 psi so that the water could be preserved in liquid state.

2.3 Determination of selected ions

Extracts from the placenta samples were subjected to a chromatographic analysis with the use of ion chromatography. Table 1 collates the conditions for the ion--chromatographic separation of selected inorganic ions in extracts from the placenta samples obtained after accelerated solvent extraction.

2.4 Analytical method validation parameters

Table 2 sets out the validation parameters for the analytical procedure to determine thiocyanate and other marker ions using ion chromatography in samples of placenta collected from various donors. The calibration curve was plotted on the basis of aqueous solutions of standards, since no reference material for the biological fluid examined was available.

Table 1
Conditions and validation parameters for the ion-chromatographic separation of analytes present in placenta samples.

	Anions	Cations
Analyte	SCN^-, F^-, Cl^-, NO_2^- , NO_3^- , PO_4^{3-} , SO_4^{2-}	Na^+, NH_4^+ , K^+, Ca^{2+}
Column	Ion Pac AS22 Analytical 2×250 mm	Ion Pac CS16 Analytical 3×250 mm
Type of suppression	conductivity	conductivity
Suppressor	ASRS-300 2-mm	CSRS-300 2-mm
Current	8 mA	36 mA
Eluent	4.5 mM CO_3^{2-} , 1.4 mM HCO_3^-	30 mM CH_3SO_3H
Elution	isocratic	isocratic
Flow rate	0.30 ml/min	0.36 ml/min

Table 2
Validation parameters of the ion chromatographic technique used to determine levels of thiocyanate and other marker ions in samples of nasal discharge.Extended uncertainty is 10 % for all analytes.

Analyte	Na^+	NH_4^+	K^+	Ca^{2+}	F^-	Cl^-	NO_3^-	NO_2^-	PO_4^{3-}	SO_4^{2-}	SCN^-
Precision [%]	0.95	1.12	0.98	0.98	1.11	0.86	0.92	1.72	1.52	1.25	1.7
Linearity (R^2)	>0.999	0.987	>0.999	0.992	0.990	0.999	>0.999	0.989	0.997	0.988	0.998
LOD [mg/l]	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.02

3. Results and discussion

During research 392 placenta samples from women who agreed to take part in the study were analyzed. In active smokers group was 16% of them. In the samples, thiocyanate ion and other inorganic ions were marked.

3.1 Levels of ions determined in placenta samples

The concentration of thiocyanate ion marked in the placenta samples of smoking women exceeded the average concentration of that ion marked in the placenta samples of non-smoking women and of passively smoking. The result are collected in Table 3. In the samples taken from active smokers concentration of fluoride, chloride, phosphate and sulphate was higher in comparison to the concentration of these ions determined in samples taken from donor from other groups.

3.2 Correlations between the levels of different analytes in nasal discharge

To test the strength of the linear relationship between the levels of the various ions present in the human placenta samples, Pearson's correlation coefficients were calculated. Those parameters enable one to define the effect of the concentration of one ion on the level of another. The values of Pearson's correlation coefficients were calculated for the obtained results of marking particular ions in the placenta samples. In Table 4 information about calculation of Pearson's correlation coefficient for ions determinate in active smokers are collected. In the group of active smokers the highest numerical values of the correlation coefficient was calculated (> 0.9) between the concentrations of Na^+ and Ca^{2+} and Mg^{2+}; NH_4^+ and Ca^{2+}. A significant numerical value of the correlation coefficient is calculated also

Table 4
The results of that calculation of Pearson's correlation coefficient .

	F^-	Cl^-	NO_2^-	NO_3^-	PO_4^{3-}	SO_4^{2-}	SCN^-	Na^+	NH_4^+	K^+	Ca^{2+}
F^-	1.00										
Cl^-	0.55	1.00									
NO_2^-	–0.23	–0.12	1.00								
NO_3^-	0.13	0.24	–0.01	1.00							
PO_4^{3-}	**0.58**	0.52	–0.18	0.20	1.00						
SO_4^{2-}	0.37	0.42	–0.06	0.01	–0.11	1.00					
SCN^-	**0.54**	0.18	–0.12	0.08	0.41	0.30	1.00				
Na^+	–0.28	–0.18	0.02	–0.12	–0.19	–0.23	–0.18	1.00			
NH_4^+	–0.22	–0.21	–0.05	–0.10	–0.16	–0.20	–0.15	0.95	1.00		
K^+	–0.51	–0.31	0.19	–0.15	–0.36	–0.30	–0.24	0.27	0.18	1.00	
Ca^{2+}	–0.21	–0.20	–0.07	–0.10	–0.14	–0.19	–0.15	**0.94**	**0.99**	**0.99**	1.00

Table 3
Information concerning the results obtained during determination selected ions in human placenta samples.

	F^-	Cl^-	NO_2^-	NO_3^-	PO_4^{3-}	SO_4^{2-}	SCN^-
Active smokers							
Standard deviation	2.187	1.677	1.526	0.202	6.069	0.928	1.344
Concentration range [mg/g]	<*LOD*–9.133	0.858–7.333	<*LOD*–10.207	<*LOD*–1.293	<*LOD*–34.477	<*LOD*–4.330	<*LOD*–6.442
Average [mg/g]	1.784	3.716	0.451	0.075	4.087	0.752	0.737
f [%]	73	98	14.4	62	97	94	98
Passive smokers							
Standard deviation	1.085	1.562	1.646	0.272	2.713	0.432	0.566
Concentration range [mg/g]	<*LOD*–5.102	<*LOD*–9.741	<*LOD*–10.203	<*LOD*–1.945	<*LOD*–10.808	<*LOD*–2.599	0.002–4.285
Average [mg/g]	0.706	2.992	0.610	0.064	2.544	0.372	0.165
f [%]	60	97	27	58	13.5	91	100
Control Group							
Standard deviation	0.945	1.672	0.823	0.506	1.491	0.371	0.015
Concentration range [mg/g]	<*LOD*–6.712	<*LOD*–12.325	<*LOD*–5.676	<*LOD*–7.638	<*LOD*–10.407	<*LOD*–2.524	<*LOD*–0.067
Average [mg/g]	0.434	2.438	0.215	0.064	1.358	0.227	0.008
f [%]	70	99	14	38	93	92	31.6

	Na^+	NH_4^+	K^+	Ca^{2+}
Active smokers				
Standard deviation	4.425	12.633	0.874	0.721
Concentration range [mg/g]	0.392–27.608	<*LOD*–59.610	<*LOD*–3.410	0.011–3.815
Average [mg/g]	2.157	4.531	0.626	0.220
f [%]	100	98	70	100
Passive smokers				
Standard deviation	0.918	1.169	0.864	0.033
Concentration range [mg/g]	0.2289–5.200	0.082–5.777	<*LOD*–3.906	0.005–0.160
Average [mg/g]	1.023	1.409	0.743	0.045
f [%]	100	100	94	100
Control Group				
Standard deviation	6.661	19.749	5.311	1.861
Concentration range [mg/g]	<*LOD*–46.517	<*LOD*–81.436	<*LOD*–81.873	<*LOD*–20.557
Average [mg/g]	4.535	12.366	1.220	0.727
f [%]	99	99	93	99

for the content of F^- and SCN^- ions in the samples of active smokers. In a group of passive smokers highest numerical values of the correlation coefficient is calculated between the concentrations of Na^+ and NH_4^+ , and K^+; and between NH_4^+ and K^+.

4. Conclusions

The conducted studies can confirm the influence of tobacco smoking on the level of concentration of those contaminants, and the obtained results form a basis for the following conclusions. A placenta is a good biological material for the evaluation of the exposure of a woman and a fetus to toxic substances during pregnancy. The technique of accelerated solvent extraction used at the stage of preparing a placenta sample has many advantages: it enables the extraction of half-solid samples like placenta, the extraction of high humidity samples, the placement in an extraction container of a very small amount of a solid sample, the use of water solutions as solvents. Thiocyanate ions are a very good biomarker of the exposure to the ingredients of tobacco smoke because they have a relatively long half-life, and differences between the concentrations of those ions among the various donor groups can be observed. The highest concentrations of thiocyanate ions in the case of placenta samples were detected in the group of actively smoking women.

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A rapid differentiation of ephedrine and pseudoephedrine by differential mobility spectrometry

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alpha function
compensation voltage
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pseudoephedrine

Abstract
The work deals with fast differentiation of ephedrine and pseudoephedrine using differential mobility spectrometry. Each target molecule appears at a specific compensation voltage under fixed radiofrequency voltage which enables differentiation of measured narcotics. Compensation voltage of product ion peak at fixed radiofrequency of 1480 V were –4.89 V for pseudoephedrine and –3.02 V for ephedrine. Calculation of specific alpha coefficients (α_2 and α_4) using averaging and least squares method to obtain alpha shapes of ephedrine and pseudoephedrine was performed. Differential mobility spectrometry has proved to be a useful analytical technique for differentiation of ephedrine and pseudoephedrine in 100 s.

1. Introduction

Identification of narcotic is an important role for toxicological laboratories (both clinical and forensic). Ephedrine and his derivatives are sympathomimetic amines frequently abused by some athletes to improve physical performance in sports. Ephedrine is mentioned in the list of prohibited substances in doping control for his stimulating effect [1]. Ephedrine and pseudoephedrine belongs to starting components in synthesis of methamphetamine. Ephedrine exists in four stereoisomers due to his two stereogenic carbons. Ephedrine and pseudoephedrine are diastereoisomers having identical fragmentation pattern, the same value of mass to charge ratio (*m/z*) and same gas chromatograms. Conventional analytical techniques like gas chromatography (GC) or electrospray mass spectrometry (ESI-MS) can not differentiate diastereoisomeric compounds [2, 3]. Ephedrine and pseudoephedrine were analysed by capillary electrophoresis [4], liquid chromatography coupled with MS [4], GC-MS with stereospecific

derivatization [6], ion mobility spectrometry [7] or high field asymmetric waveform ion mobility spectrometry (FAIMS) coupled with MS [8].

FAIMS or differential mobility spectrometry (DMS) belongs to high fielded ion mobility spectrometry techniques. Electric field is applied by separation voltage (radiofrequency voltage) and compensation voltage. The electric field applied to DMS/FAIMS can be half sinusoidal, bisinusoidal or rectangular. The field dependence is characterized by alpha function which is defined for bisinusoidal waveform as [9]:

$$-CV = \left(CV^3 + \frac{5}{6}CV \cdot RF^2 + \frac{1}{9}RF^3\right)\alpha_2 + \\ +\left(CV^5 + \frac{25}{9}CV^3 \cdot RF^2 + \frac{10}{9}CV^2RF^3 + \frac{55}{72}CV \cdot RF^4 + \frac{55}{486}RF^5\right)\alpha_4 \quad (1)$$

where *CV* is compensation voltage at specific radiofrequency voltage (*RF*) and α_2 and α_4 are parameters of ion behaviour at high field strength. Parameters α_2 and α_4 are only unknown parameters which can be calculated by averaging or least squares methods as a system of equations.

The main goal of this work is a fast differentiation of ephedrine and pseudoephedrine using DMS and comparison of averaging and least squares method for calculation of alpha coefficients.

2. Experimental

2.1 Reagents and chemicals

HPLC-grade methanol was purchased from Sigma-Aldrich. Samples of ephedrine and pseudoephedrine were purchased from Institute of Criminalistics Prague, Czech Republic. A volume of 1 μL of each narcotic (1000 ng μL^{-1} diluted in methanol) were injected via a syringe to vapor phase generator.

2.2 Instrumentation

A prototype of DMS analyser with ^{63}Ni as an ionization source was used for ephedrine and pseudoephedrine analysis. Separation voltage was applied from 500 to 1500 V. Radiofrequency step size was 5 V with 200 radiofrequency steps. Values of compensation voltage were applied in range from −40 to +15 V in 0.28 V steps. Dried air (purified by activated carbon and dewatered by molecular sieve) was used as a carrier gas. Flow rate of carrier gas was 50 ml min^{-1} and temperature was controlled at 90 °C. Calculation of alpha coefficients and drawing an alpha shapes were effected in MATLAB 2015b software.

Table 1
Values of radiofrequency voltage and compensation voltage for ephedrine and pseudoephedrine.

Compound	*RF* [V]	*CV* [V]
Ephedrine	1180	–2.45
	1280	–2.61
	1380	–2.89
	1480	–3.02
Pseudoephedrine	1180	–3.68
	1280	–4.15
	1380	–4.55
	1480	–4.89

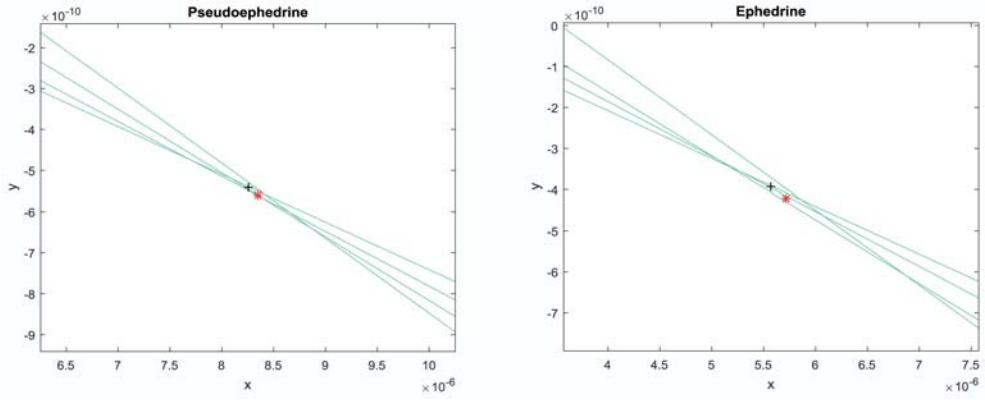

Fig. 1. Illustration of calculation of alpha coefficients using averaging methods (star) and least squares methods (cross) for pseudoephedrine and ephedrine.

3. Results and discussion

Using several values of compensation voltage at several different radiofrequency equation (1) can be solved by averaging or least squares methods to find the value of alpha coefficients. Averaging method exploits average of all combination of CV/RF sets to presumption of alpha coefficients. The method least squares means that the overall solution minimizes the sum of the squares of the errors made in the results of every single equation. This method is a standard method in regression analysis.

For ephedrine and pseudoephedrine we used four values of compensation voltage from radiofrequency 1080 V to 1480 V as seen in Table 1. Each set of RF/CV are illustrated by straight line in Fig. 1. Intercept point of these straight line is represented by red star for averaging methods and black cross for least squares methods. Values of α_2 and α_4 coefficients, and differences between these methods for ephedrine and pseudoephedrine, are summarized in Table 2. Alpha coefficients are instrumental to construct alpha shape which is used for identification of measured ions. Alpha shapes were constructed for both averaging and least

Table 2
Values of α_2 and α_4 coefficients using averaging (AVG) or least squares (LS) methods, and difference (Δ) between AVG and LS methods.

Compound	α_2 [10^{-6}]			α_4 [10^{-10}]		
	AVG	LS	Δ	AVG	LS	Δ
Ephedrine	5.7183	5.5731	0.1452	–4.2051	–3.9316	0.2735
Pseudoephedrine	8.3539	8.2526	0.1013	–5.6011	–5.4128	0.1883

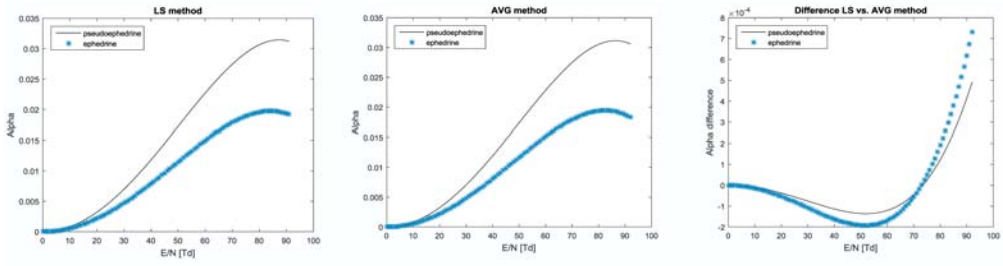

Fig. 2. Alpha shape of ephedrine and pseudoephedrine using averaging (AVG) and least squares (LS) method and differences between these methods.

squares methods (Fig. 2). At 50 and 90 Td (1 Td = $1 \cdot 10^{-17}$ V cm^2) were found the biggest differences between AVG and LS method as.

Narcotics were measured in positive channel because of their high proton affinities. High-energy beta particles of ^{63}Ni initiate ionization process through collision with carrier gas. As a result of this process reactant ions are formed. These low-energy reactant ions interact with analyte vapours to produce product ions. Alpha shapes as well as differential mobility spectrum (Fig. 3) enable

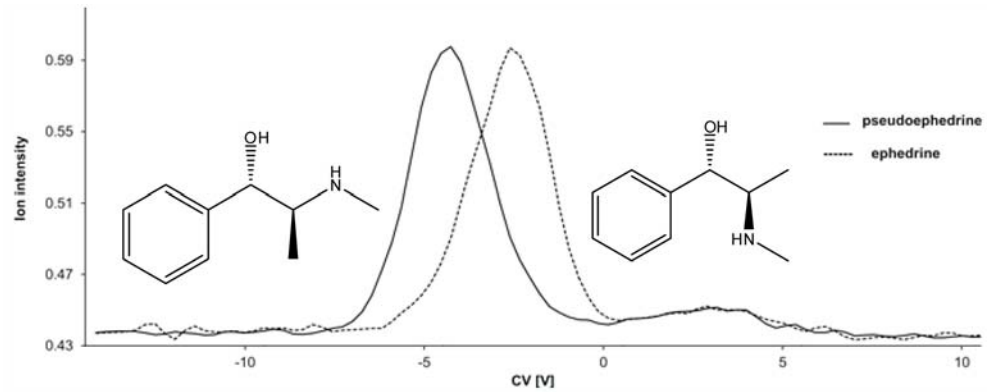

Fig. 3. Differential mobility spectrum of pseudoephedrine and ephedrine at radiofrequency 1480 V at 90 °C.

differentiation of ephedrine and pseudoephedrine. Differential mobility spectrum of ephedrine and pseudoephedrine was measured at radiofrequency 1480 V, where the difference between these compounds were the most significant. Compensation voltage of product ion peak of pseudoephedrine and ephedrine at radiofrequency 1480 V were –4.89 and –3.02 V, respectively.

4. Conclusions

Ephedrine and pseudoephedrine appears at a specific compensation voltage under fixed rediofrequency. This finding enables detection and also differentiation of these diastereoisomeric compounds. Comparison of alpha shapes and specific alpha coefficients for averaging and least squares method were drawn and calculated for ephedrine and pseudoephedrine.

Acknowledgments

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Quantification of ethosuximide in human serum by GC-MS after butyl chloroformate derivatization

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Keywords
chloroformate
ethosuximide
GC-MS
serum

Abstract
A simple, sensitive and robust method for determination of antiepileptic drug ethosuximide in human serum using GC-MS was developed and validated for clinical toxicology purposes. This method employs an emerging class of derivatization agents: alkyl chloroformates allowing the efficient and rapid derivatization of both the amino and carboxylic groups of the tested antiepileptic drug within seconds. The derivatization protocol was optimized using the Design of Experiment, and the entire sample preparation requires less than 5 minutes. Linear calibration curves were obtained in the concentration range 0.5–150 μg/mL, with adequate accuracy (98.0–109.0%) and precision (< 10.4%). The method was successfully applied to quantification of ethosuximide in the serum of patients in both therapeutic and toxic concentration ranges

1. Introduction

Ethosuximide (3-ethyl-3-methylpyrrolidine-2,5-dione) is the most effective succinimide antiepileptic drug and has received wide use for treatment of absence seizures [1]. Treatment with ethosuximide requires long-term therapy and therapeutic blood concentration range of ethosuximide is relatively high 40–100 μg/mL [2]. The monitoring of ethosuximide concentration in blood is required for seizure control and reducing the possibility of overdose [3].

A number of laboratory methods have been described for determination of antiepileptic drugs in biological materials including immunoassay [4], high-performance liquid chromatography [5, 6], gas chromatography [7, 8] or electrokinetic capillary methods [1]. In the past decade, LC-MS has become the method of choice for determining antiepileptics [9, 10], although GC-MS methods have been occasionally reported as well [11]. Previously published methods

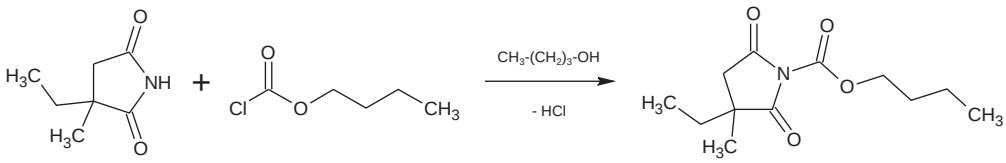

Fig. 1. Scheme of derivatization reaction of ethosuximide with butyl chloroformate / *n*-butanol.

employed various sample preparation techniques, such as solid-phase extraction [9], liquidliquid extraction [11] or derivatization [7, 12]. In our case, alkyl chloroformate was used as suitable derivatization reagents due to their high reactivity, high recovery and ability to react in aqueous media within seconds. Furthermore, it is able to react with molecules possessing an active hydrogen, e. g. carboxylic acids, phenols and amines, yielding the corresponding derivatives (carboxylic acid esters, carbonic acid diesters and carbamates). This fact makes it convenient reagent for derivatization of a broad range of compounds, such as organic and amino acids [13, 14], steroids [15] and various drugs [16, 17].

This study was performed to develop a simple, sensitive and robust method for determining ethosuximide in human serum using GC-MS. Sample preparation was partly based on an alkyl chloroformate derivatization study by Kostić [18] and on our previously research in this field [19]. A general scheme of the derivatization reaction is shown in Fig.1. The optimum conditions for derivatization have been found by using the chemometric approach Design of Experiment [20, 21], replacing the common one-factor-at-a-time procedures because they are not time--effective and do not take into account possible interactions among the individual parameters.

2. Experimental

2.1 Reagents and chemicals

Ethosuximide, butyl chloroformate (98%), 3-amino-3-(4-chlorophenyl)propionic acid (97%), pyridine (99.8%) and sodium acetate (99 %) were purchased from Sigma-Aldrich. Sodium hydroxide, acetic acid, and *n*-butanol, all analytical quality (p.a.), were purchased from Penta (Prague, Czech Republic). Blank human serum was purchased from ACQ Science GmbH (Rottenburg-Haifinger, Germany).

2.2 Sample pretreatment

A volume of 200 µL of serum were mixed with 200 µL of 1 mol/L sodium hydroxide solution (pH ≈ 12) in a 1.5 mL Eppendorf tube. Then 25 µL of internal standard solution (3-amino-3-(4-chlorophenyl)propionic acid, c = 31 µg/mL in water), 100 µL of *n*-butanol and 50 µL of pyridine were added and the mixture was shaken

for 10 s. Finally, 50 μL of butyl chloroformate was added and the mixture was sonicated for 1 min. Then the sample was centrifuged for 30 s (ca. 9 600 g) and 80 μL of upper butanol layer was transferred into 400 μL glass insert placed in 1.5 mL glass vial, and the vial was gas-tight crimped.

2.3 Instrumentation

GC-MS analyses were performed using a GC7860/5742C MSD instrument (Agilent Technologies) equipped with an HP5-MS fused silica column (30 m × 0.25 mm ID, 0.25 μm film thickness; Agilent Technologies). Helium (purity 99.9992 %, Air Product, Prague, Czech Republic) was employed as carrier gas. The oven temperature was held at 85 °C for 2 min following injection and raised to 170 °C at 30 °C min^{-1} and then to 300 °C at 15 °C min^{-1}, total run time 18.5 min. The instrument parameters were as follows: inlet temperature 270 °C, transfer line 300 °C. All the injections were performed in the splitless mode (1 min) and with an injected sample volume of 1 μL. The mass spectrometer was operated with electron impact ionization (70 eV) and the selected ion monitoring mode was used for quantification (quantifier/qualifiers *m/z*): ethosuximide (213/70, 113, 142), 3-amino-3-(4-chlorophenyl)propionic acid (254/140, 240, 355).

2.4 Statistical optimization of the derivatization protocol

The construction and analyses of the Design of Experiment procedure were carried out using the Minitab 16 statistical package (Minitab Inc., USA). The derivatization parameters and their levels for optimization (pH value, volumes of butyl chloroformate, pyridine and butanol, sonication time) were selected on the basis of the author's experience (Table 1). The maximum response, defined as the absolute area of derivatized ethosuximide, was sought. Face-centered central composite design was employed to find the optimal values of these parameters for the derivatization protocol. The Minitab 16 software generated 18 experimental runs, combining the selected parameters and their individual levels. The results

Table 1
Parameters and their levels for statistical optimization of butyl chloroformate derivatization.

Parameter	Level		
	Low	Central	High
pH	4	7	12
Volume of butyl chloroformate (μL)	50	85	120
Volume of pyridine (μL)	30	65	100
Volume of butanol (μL)	100	200	300
Sonication time (min)	1	3	5

Table 2
Within-day and between-day method precision and accuracy of ethosuximide in serum (six days, six replicates).

	$c_{nominal}$ (μg/mL)	$c_{measured}$ (mean ± SD)	Precision (RSD, %)	Accuracy (%)
Within-day	0.5	0.49 ± 0.05	10.4	98.0
	5.0	5.15 ± 0.39	7.6	103.0
	50.0	52.6 ± 4.5	8.6	105.2
Between-day	0.5	0.50 ± 0.04	7.8	99.0
	5.0	5.45 ± 0.48	8.8	109.0
	50.0	51.4 ± 3.2	6.2	102.7

were evaluated using the ANOVA test. Optimal conditions for ethosuximide derivatization were predicted by using the desirability function to find the combination of parameters which yields the maximum response value.

3. Results and discussions

3.1 Analytical performance

Calibration serum samples were prepared using blank human serum (180 μL) spiked with 20 μL of the appropriate aqueous stock solutions containing ethosuximide to yield a concentration range within the 0.5–150 μg/mL range (0.5; 1; 2; 5; 10; 50; 100 and 150 μg/mL) and processed as described in the Sample pretreatment section. The calibration dependence in a given range was demonstrated to be linear and the coefficient of determination was 0.9992 in the serum. The limits of detection (LOD) and quantification (LOQ) were arbitrarily set to 0.5 μg/mL [22]. At this concentration level, the precision and accuracy were 10.4% and 98.0% for serum samples, respectively [22].

The precision of the developed method (Table 2), expressed as the relative standard deviation (RSD), was evaluated by analyzing six individual blank serum samples containing ethosuximide at concentrations of 0.5, 5 and 50 μg/mL on the same day (within-day precision) as well as on six different days (between-day precision). The RSD obtained was found to be satisfactory with a value of less than 15 % in all cases and the method performed well with adequate accuracy (98.0 109.0 %).

The selectivity was evaluated according to Peters [23] by analyzing ten different blank human serum samples collected from volunteers; no signals interfering with the signal of the analyte or the IS were recorded. Carryover was assessed by measuring three samples (200 μg/mL) with subsequent matrix blank sample measurements and the method is free of carryover at this concentration. The post

preparation stability of the derivatized samples in the autosampler has been evaluated (50 μg/mL, three replicates) for up to 48 hours and the accuracy was still within the acceptance criteria (± 20%).

3.2 Application of the method

The newly developed method has been established as a routine analytical tool for quantification of ethosuximide in our laboratory, mainly for therapeutic drug monitoring an concentrations in the range of 5.2–102.0 μg/mL of ethosuximide in serum (n = 5).

4. Conclusion

This paper describes a newly developed GC-MS method for determination of ethosuximide in human serum after butyl chloroformate derivatization. The derivatization protocol was optimized using the Design of Experiment and the entire sample preparation is complete within 5 minutes, which is suitable for time--effective support of a clinical diagnosis. The developed method has been used for quantification of ethosuximide in serum for therapeutic drug monitoring. Furthermore, the wide calibration range of the method also makes it suitable for determination of ethosuximide in cases of severe drug overdoses.

Acknowledgments

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Voltammetric determination of 1-hydroxy-*N*--(4-nitrophenyl)naphthalene-2-carboxamide by voltammetry at a glassy carbon electrode in microvolumes of dimethyl sulfoxide

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Keywords
differential pulse voltammetry
dimethyl sulfoxide
glassy carbon electrode
1-hydroxy-*N*-(4-nitrophenyl)naphthalene-2-carboxamide
square wave voltammetry

Abstract
Voltammetric reduction and oxidation of 1-hydroxy-*N*-(4-nitrophenyl)naphthalene-2-carboxamide was investigated at glassy carbon electrode in dimethyl sulfoxide. Cyclic voltammetry was used to investigate the mechanism of reduction of nitro group and oxidation of hydroxyl group. The analyte was successfully determined in dimethyl sulfoxide by differential pulse voltammetry and the whole voltammetric procedure was miniaturised. Square wave voltammetry was employed to reduce the interference from dissolved oxygen. Determination in one drop (20 μL) of 0.1 mol L^{-1} tetrabutylammonium tetrafluoroborate in dimethyl sulfoxide provided very similar results compared to determination in the bulk solution. Limits of quantification were 5.0 μmol L^{-1} for cathodic voltammetry and 5.3 μmol L^{-1} for anodic voltammetry.

1. Introduction

Traditional antimicrobial chemotherapeutics are becoming less and less efficient in treatment of current infectious diseases. The direct consequence is a new rise of infections such as tuberculosis caused by *Mycobacterium tuberculosis* and other diseases caused by non-tuberculous mycobacteria. These pathogens are responsible for respiratory, gastrointestinal, skin or bone infections. Increasing number

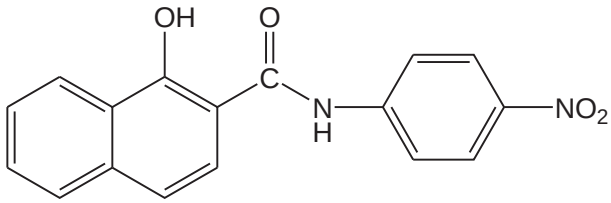

Fig. 1. Chemical structure of 1-hydroxy-*N*-(4-nitrophenyl)naphthalene-2-carboxamide.

of infections and resistance of pathogens to clinically used drugs makes the discovery of new molecular scaffolds a priority to achieve effective control of these bacterial strains. The substance investigated in this contribution belongs to a group of those new chemotherapeutics. They were studied for their antimycobacterial efficiency in papers [1–3], their activities were compared to clinically used drugs and some of those compounds could be considered as promising agents for subsequent design of novel antimycobacterial agents. This particular substance, 1-hydroxy-*N*-(4-nitrophenyl)naphthalene-2-carboxamide (HNN, Fig. 1), belongs to a group of 1-hydroxynaphtalene-2-carboxamides and was chosen as a model substance.

The aim of this work is to determine HNN by voltammetric methods that provide an easy, fast and reliable means of determination. Mechanisms of electrode reduction and oxidation can be analogical to those occurring in living organism providing valuable biochemical/metabolic information. Since HNN has never been studied by methods of electroanalytical chemistry, no information concerning the mechanism could be found. It was then necessary to study the mechanism of substructures or similar structures and based on those information suggest the mechanism of HNN electrode reactions. HNN contains reducible nitro group studied extensively by polarography or voltammetry [4–6] that is reduced in two steps in non-aqueous solvents. The first reduction step results in an intermediate product, anion-radical that is reduced in the second step at more negative potentials to hydroxylamine. HNN also contains oxidizable hydroxyl group [7, 8], in which case a sterically favourable interaction between oxygen and nitrogen in amide group could lead to a cyclisation of a final product.

As it is presumed that HNN or other above-mentioned compounds will be determined in matrices whose amount and volume is very limited, this paper is focussed on miniaturisation of the whole method suitable for the determination in volumes in range of microliters.

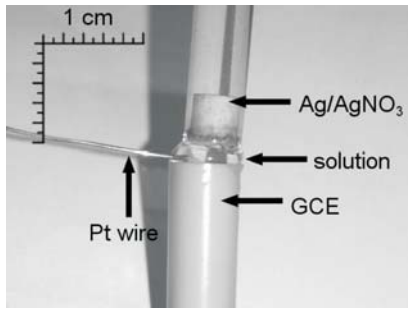

Fig. 2. Instrumentation for measurements in a drop of solution containing working GCE, $Ag/AgNO_3$ reference electrode and platinum wire auxiliary electrode.

2. Experimental

2.1 Reagents and chemicals

A stock solution of HNN (CAS number: 68352-27-2; Sigma Aldrich) was prepared by dissolving the substance in dimethyl sulfoxide (DMSO, Penta, Czech Republic). Tetrabutylammonium tetrafluoroborate (p.a., Sigma Aldrich) was used as a supporting electrolyte in DMSO. All chemicals were used without further purification.

2.2 Instrumentation

Voltammetric measurements were carried out in a three-electrode system with working glassy carbon disc electrode (GCE, 2 mm diameter, Metrohm, Switzerland), auxiliary platinum wire electrode (Eco-Trend Plus, Czech Republic) and a reference electrode $Ag/AgNO_3$ (0.01 mol L^{-1}), Bu_4NBF_4 (0.1 mol L^{-1}) in DMSO. Instrumentation for measurements in a drop is shown in Fig. 2. GCE was polished prior to measurements with aqueous slurry of alumina powder (1.1 μm) to mirror-like appearance. Cyclic voltammetric (CV) measurements were usually carried out at a scan rate 100 mV s^{-1}. Differential pulse voltammetry (DPV) was carried out using scan rate 20 mV s^{-1}, pulse width 100 ms and pulse amplitude value of 50 mV. Square wave voltammetry (SWV) was carried out at frequency 100 Hz, pulse amplitude –50 mV and step –4 mV. CV and DPV measurements were carried out on Eco-Tribo Polarograph controlled by Polar Pro 5.1 software (both Polaro-Sensors, Czech Republic); SWV was carried out on Autolab PGSTAT101 (Metrohm) controlled by Nova 1.11.2 software (Metrohm).

3. Results and discussion

Mechanisms of reduction and oxidation of HNN on the electrode surface were at first studied by cyclic voltammetry in 10 mL volume and compared with mechanisms suggested in papers quoted in the introduction. HNN provided three reduction responses. The first observable signal at –1.4 V should correspond to one-electron reversible reduction to anion-radical. The second signal at more negative potential (–2.5 V) should correspond to the reduction of anion-radical to

Table 1
Parameters of calibration curves of 1-hydroxy-*N*-(4-nitrophenyl)naphthalene-2-carboxamide determination measured on GCE in 0.1 mol L^{-1} Bu_4NBF_4 in DMSO in a drop (20 µl) of solution.

Method	Measured peak [V]	Conc. range [µmol L^{-1}]	Slope [mA $mol^{-1}L^{-1}$]	Intercept [nA]	R^2	*LOQ* [µmol L^{-1}]
DPV (without O_2)	–1.3	2–10	–15.7	+4.08	0.9960	5.0
		10–100	–5.32	–11.8	0.9922	–
DPV (with O_2)	+0.4	2–10	+1.94	+1.62	0.9854	5.3
		10–100	+1.57	–6.39	0.9934	–
	–1.3	40–100	–5.51	+114	0.9442	60
SWV (with O_2)	–1.3	2–10	–129	+657	0.9888	7.3
		10–100	–28.1	–758	0.9945	–

hydroxylamine. The third signal at –1.1 V could not be identified. One of the explanations is that it could correspond to the reduction of nitro group in the presence of trace amounts of water. Only one oxidation signal at +0.5 V was observed.

Concentration dependencies were measured in 0.1 mol L^{-1} Bu_4NBF_4 in DMSO by DPV method. HNN was determined by both cathodic and anodic voltammetry. Limits of quantification obtained by these methods were 5.0 µmol L^{-1} (reduction) and 5.3 µmol L^{-1} (oxidation). Calibration curves were not linear in the whole studied range, but they could be divided into two linear sections.

Afterwards, this procedure and instrumentation (Fig. 2) was downscaled and volumes of measured solutions were decreased to 20 µL. A peak of dissolved oxygen in cathodic range turned out to be the main problem, because it was greatly interfering with the peak of HNN and consequently a limit of quantification rose about ten times. Method of SWV was used to eliminate this problem. After an optimization of this method it was possible to improve response ratio of HNN to oxygen. Limit of quantification was significantly decreased. Further decrease of *LOQ* could be achieved by DPV method and removal of the dissolved oxygen by putting the whole instrumentation with a drop of solution into the stream of nitrogen for 10–15 min. To conclude, DPV method was found to have lower *LOQ*, but a time consuming removal of the dissolved oxygen is necessary. On the other hand, SWV is a faster method but with a higher *LOQ*. Additional measurements by SWV in solutions without dissolved oxygen provided same results. Results for both reduction and oxidation responses are summarised in Table 1 and voltammograms with a calibration curve in one drop are illustrated in Fig. 3.

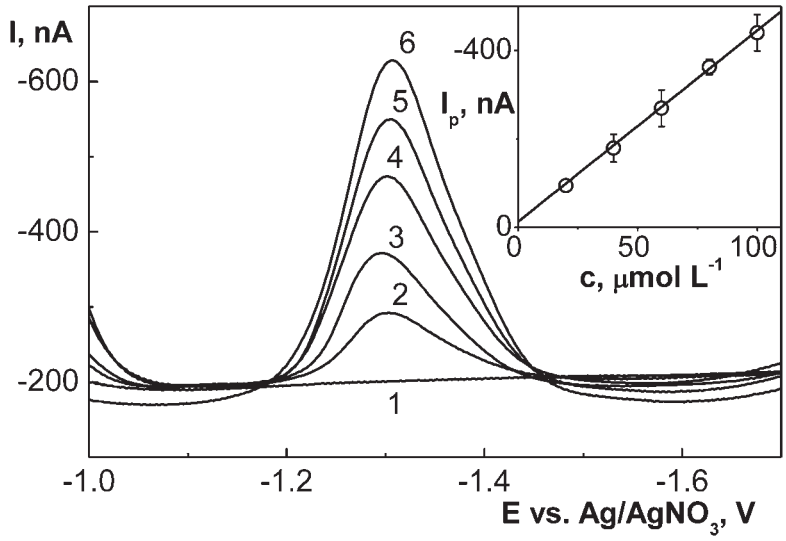

Fig. 3. DP voltammograms of 1-hydroxy-*N*-(4-nitrophenyl)naphthalene-2-carboxamide at concentrations of (1) 0.0; (2) 20; (3) 40; (4) 60; (5) 80; and (6) 100 μmol L^{-1} obtained on the GCE in one drop (20μl) of 0.1 mol L^{-1} Bu_4NBF_4 in DMSO after the removal of dissolved oxygen. Calibration curve is shown in the inset.

4. Conclusions

Mechanism of reduction and oxidation of HNN was proposed. Results are in agreement with studies about substructures found in literature, but further investigations are necessary. Determination of HNN in a single drop of DMSO (20 μL) by both cathodic and anodic voltammetry provided favourable results, comparable to the determination in a bulk solution.

Acknowledgments

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LC enantioseparation of selected enantiomers using novel chiral selectors

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Keywords
cyclofructan
enantioseparation
HPLC

Abstract
An HPLC method was developed for enantiomeric separation of new chiral compounds with potential biological activity on three cyclofructan based chiral stationary phases. A number of experimental parameters, which influence the separation of analytes in normal chromatographic mode were investigated. The separation conditions were optimized and the thermodynamic study was carried out for selected analytes.

1. Introduction

Cyclofructans (CF) are relatively new and unique group of chiral selectors. They belong to the macrocyclic oligosaccharides. Native cyclofructans showed limited enantioselectivity. Derivatized cyclofructans, CF6 and CF7, with a high degree of selectivity toward a variety of chiral compounds have been used as chiral stationary phases (CSPs) in different separation techniques [1].

Phytoalexins are structurally diverse group of natural secondary metabolites produced by plants in response to various forms of stress and they represent a specific group of phytoalexins due to their antiproliferative and anticancer activity and they are attractive lead compounds for anticancer drug development [2].

In this work, the comparison of enantioseparation of novel racemic analogs of phytoalexin spirobrassinin under normal phase conditions on three derivatized cyclofructan chiral stationary phases, CF-RN, CF-P, and CF-DMP were performed. The effect of the column temperature on the retention and enantioselectivity of racemic phytoalexins was explored.

2. Experimental

2.1 Reagents and chemicals

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

2.2 Instrumentation

HPLC experiments were performed on Infinity 1260 HPLC system with an injection valve Rheodyne with a 20 μL sample loop and an LC UV-visible detector with variable wavelength. The collection and evaluation of data was carried out using Agilent ChemStation software.

The chromatographic columns CF-RN (Larihc CF6-RN), CF-P (Larihc CF6-P) and CF-DMP (Larihc CF7-DMP) (250×4.6 mm, particle size 5 μm) were obtained from Department of Chemistry and Biochemistry, University of Texas at Arlington, USA.

3. Results and discussion

Enantioseparation of novel racemic analogs of the natural phytoalexins was performed on three different cyclofructan chiral stationary phases, CF-RN, CF-P, and CF-DMP under normal phase conditions. The mobile phase consisted of *n*-hexane and polar modifier, in some cases we added small amount of additives. The results obtained on three different cyclofructan chiral stationary phases were evaluated and compared. The influence of varying column temperature on retention and resolution of the enantiomers was also studied.

4. Conclusions

Three different derivatized cyclofructan chiral stationary phases were evaluated for the HPLC enantioseparation of new chiral spirobrassinin analogs under normal phase mode. The results acquired were evaluated and compared. A comparison of the chiral recognition abilities of cyclofructan-based chiral stationary phases showed that the RN-CF6 provides better stereoselective separation ability towards racemic mixtures of spirobrassinin analogs. The thermodynamic study showed that the chiral separation was enthalpy controlled on all cyclofructan columns.

Acknowledgments

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Arsenic speciation by hydride generation coupled with atomic fluorescence spectrometry

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Keywords
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HPLC
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speciation analysis

Abstract
The aim of this work is to present methods for determination of arsenic by hydride generation (HG) and detection by atomic fluorescence spectrometry (AFS). First part is dedicated to speciation analysis by high performance liquid chromatography and postcolumn HG-AFS. Second part is dedicated to identification of volatile products of methylated arsenic species (methylarsonate, dimethylarsinate, and trimethylarsane oxide). It was found that the structure of resulting arsanes strongly depended on the concentration and type of acid. Third part is dedicated to possibility of isolation of arsenosugars from algae and their HG activity.

1. Introduction

The abundance of arsenic in the earth's crust is on average concentration of 5 μg g^{-1}. It is a component of 245 minerals, usually associated with metals like copper, gold, lead, and zinc in sulfidic ores. Arsenic is introduced into the environment by anthropogenic and natural activities. Acute or chronic exposure to arsenic leads to serious health hazards [1].

The arsenic toxicity strongly depends on its chemical form (speciation). The inorganic species arsenite (iAs^{III}) and arsenate (iAs^{V}) are more toxic than methylated pentavalent species such as methylarsonate (MAs^{V}), dimethylarsinate ($DMAs^{V}$), and trimethylarsane oxide ($TMAs^{V}O$). There also exist nontoxic species such as arsenobetaine, arsenocholine, and potentially toxic compounds like "arsenosugars" (derivatives of dimethylarsinoylribosides). Therefore information about total arsenic concen-tration in food (especially in marine food) is not toxicologically informative [2]. The iAs^{III+V}, MAs^{V}, $DMAs^{V}$, and $TMAs^{V}O$ are "hydride active" species (they can be converted to volatile hydride), more complex arsenic

species were considered as "nonhydride active". But Schmeisser et al. [3] and Regmi et al. [4] shown that also arsenosugars are hydride active species but with low yield 5–10%.

The high performance liquid chromatography (HPLC) coupled with inductively coupled plasma mass spectrometry (ICP-MS) is the most powerful and common method for arsenic speciation analysis. However this technique is not available in many runtime control laboratories because of expensive instruments and highly skilled analysts are required [2]. The more economic approach is to substitute the ICP-MS with atomic fluorescence spectrometry (AFS). However, the analyte must be converted to the gaseous phase in order to be detectable by AFS. This is usually achieved by hydride generation (HG) used as postcolumn derivatization step. During the HG the "hydride active" arsenic species are converted to their corresponding arsanes: iAs^{III+V} to AsH_3, MAs^{V} to CH_3AsH_2, $DMAs^{V}$ to $(CH_3)_2AsH$, and $TMAs^{V}O$ to $(CH_3)_3As$. Unfortunately, the HG efficiency of the above listed species depends strongly on the concentration of acid. Therefore it is experimentally difficult to achieved high HG efficiency for all arsenic species.

A different approach to arsenic speciation analysis is based on generation of substituted arsanes. This technique consists of two steps: *(i)* generation of arsanes from arsenic species, and *(ii)* separation of generated arsanes. The explicit relation between a hydride active species and its corresponding arsane i.e. iAs^{III+V} – AsH_3; MAs^{V} – CH_3AsH_2; $DMAs^{V}$ – $(CH_3)_2AsH$; and $TMAs^{V}O$ – $(CH_3)_3As$, is a necessary condition for reaching accurate results. It was generally believed that this condition is fulfilled for HG [5].

The aim of this work is to present our published results about hyphenated technique HPLC-HG-AFS [6], demethylation effect on arsenic species during HG [7], and our preliminary results about HG of arsenosugars.

2. Experimental

2.1 Atomic fluorescence spectrometry

The in-house assembled research grade non-dispersive AFS was employed [8]. Miniature diffusion flame under optimum conditions was used as the atomizer [9].

2.2 Inductively coupled plasma mass spectrometry

An Agilent 7700x ICP-MS spectrometer equipped with an Integrated Sample Introduction System and with HPLC pump (Agilent 1200, USA) with PRP-X100 column (250×4.6 mm, 10μm particle size) were used.

2.3 Standards and reagents

Deionized water (< 0.2 μS cm^{-1}, Ultrapur, Watrex) was used for the preparation of all solutions. A stock solution of 1000 mg dm^{-3} was prepared for each arsenic species in deionized water using following compounds: iAs^{III} from As_2O_3 (Lachema, Czech Republic); iAs^{V} from As stock standard solution (Merck, Germany); MAs^{V} from $Na_2CH_3AsO_3.6H_2O$ (Chem. Service, USA); $DMAs^{V}$ from $(CH_3)_2As(O)OH$ (Strem Chemicals, USA); and $(CH_3)_3AsO$ (obtained by courtesy of Dr. William Cullen, University of British Columbia, Canada). A reducing solution of $NaBH_4$ (Fluka, Germany) in 0.1% KOH (Lach-ner, Czech Republic) was prepared fresh daily. Hydrochloric acid (Merck, Germany) was used for HG. Mobile phase for anion exchange chromatography was 20 mmol dm^{-3} phosphate buffer (from KH_2PO_4 (Merck, Germany) and K_2HPO_4 (Xenon Lodz, Poland)), pH adjusted to 5.6.

2.4 Algae extracts

Algae samples (Nori) were brought in local supermarket. About 200 mg of dried algae sample were accurately weighed into 15 cm^3 polyethylene vessels and 8 cm^3 of deionized water was added. These vessels were placed into the ultrasonic bath (Elmasonic One, Elma, Germany) for 3 hours. Supernatant was removed into 50 cm^3 polyethylene vessels and diluted up to 25 cm^3.

2.5 Hydride generators

Flow injection hydride generator is described in reference [6]. It employed two peristaltic pumps. The first one delivered reagents ($NaBH_4$, HCl, and deionised water into which an arsenic standard was dosed) into the 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.

Batch generator is detailed in reference [7]. HCl or TRIS buffer were pumped by the peristaltic pump into the GLS. HCl/TRIS channel lead through the six-port injection valve. Another peristaltic was used to introduce a solution of $NaBH_4$ into the GLS. Separate channel had been used to remove liquid waste from the GLS after the reaction was completed. The output from the GLS was connected to the cryogenic trap (CT) or directly to the T-junction serving to introduce hydrogen and argon for the atomizer.

3. Results and discussion

3.1 HPLC-hydride generation -atomic fluorescence spectrometry

Firstly, conditions of hydride generation for iAs^{III+V}, MAs^{V}, and $DMAs^{V}$ in the

flow injection mode were optimized. The dependence of HG efficiency on concentration of HCl was in good agreement with other works [10, 11]. The iAs^{III} was quantitatively converted to arsane in the whole tested range of HCl concentration, while for iAs^{V} the generating efficiency increased with higher concentration of HCl. The methylated species are easily generated at lower concentration of HCl and with higher concentration their HG efficiency decreased (especially for $DMAs^{V}$). Therefore a compromise concentration (between iAs^{V} and $DMAs^{V}$) a 2 mol dm^{-3} was chosen.

To increase the reaction time (and increase the reaction yield) the volume of the reaction coil was increased (up to 8.9 cm^{-3}). This increased generation efficiency for all species. However, for iAs^{V} and $DMAs^{V}$ it was still lower than for iAs^{III} and MAs^{V}.

The increase of concentration of $NaBH_4$ to 2.5% (m/v) leads to unification of HG efficiencies of all four arsenic species. Assuming that iAs^{III} is generated to AsH_3 quantitatively, [12] it is evident that all species are generated with 100% efficiency.

Unfortunately, the high concentration of $NaBH_4$ and high volume of reaction coil leads to high noise which dramatically increases the limits of detection (*LOD*). Therefore a new design of GLS unit was constructed. The connection with HPLC resulted in *LOD*s for iAs^{III}, iAs^{V}, MAs^{V}, and $DMAs^{V}$, respectively, 40, 97, 57, and 55 pg cm^{-3}. Accuracy of the method was verified by standard reference material NIST 2669 (ref. [6]).

3.2 Demethylation during hydride generation

The demethylation, *i.e.* formation of noncorresponding arsanes, was studied in the batch hydride generator. It was found that the demethylation of MAs^{V}, $DMAs^{V}$, and $TMAs^{V}O$ was lower at higher concentration of HCl. Unfortunately these species are not easily generated at this pH. When the $NaBH_4$ was partially decomposed (by reaction with HCl), before it reacted with arsenic species, the demethylation effect was almost completely suppressed. This led us to the assumption that demethylation is due to action of specific hydridoboron species (mainly by the first one $(OH)BH_3^-$). Similar behaviour was observed also for sulfuric and perchloric acid. For acetic acid and TRIS buffer with L-cysteine prereduction there was no demethylation observed. A strange situation was observed in the case of nitric acid, where only the demethylation of $TMAs^{V}O$ took place [7].

This demethylation effect of arsenic species can jeopardize the accuracy of arsenic determination by HG-CT technique.

3.3 Hydride generation of arsenosugars

The arsenosugars are not commercially available compounds. Therefore they must be synthesized or isolated from natural sources [13]. The algae contained

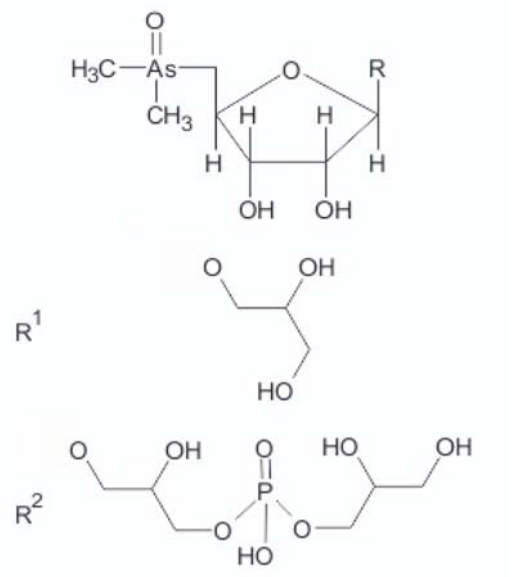

Fig. 1. Structures of two arsenosugars: R^1 = 3-[5'-deoxy-5'-(dimethylarsinoyl)-β-ribofuranosyloxy]--2-hydroxy-propylene glycol (Gly-sug); R^2= 3-[5'-deoxy-5'-(dimethylarsinoyl)-β-ribofuranosyloxy]-2-hydroxypropyl-2,3-hydroxypropyl phosphate (PO_4-sug).

a high amount of arsenic in the form arsenosugars. These species are soluble in water and can be easily extracted by ultrasonic bath. Unfortunately most of the algae contain also other arsenic species. The Nori (red algae genus Pyropia) water extracts usually contains most of the arsenic in the form of PO_4-sug (Fig. 1) [14]. In our case the Nori extract contained 95% of PO_4-sug, 3% of Gly-sug, and 1% of $DMAs^V$ (determined by HPLC-ICP-MS), with total extractable arsenic concentration of 10 mg g^{-1} (determined by ICP-MS). The total amount of arsenosugars in the extract is 98% which is acceptable purity for a "standard".

Preliminary test with extracts shows that there are no matrix effects on HG (proved by spike of $DMAs^V$) but the HG efficiency is low 10% (corrected on the amount of $DMAs^V$ in the sample) at 0.5 mol dm^{-3} HCl, 1% $NaBH_4$, and 8.9 cm^3 volume of the reaction coil.

4. Conclusion

Due to excellent sensitivity the HG-AFS is a powerful tool for speciation analysis of arsenic and/or mechanistic study on ultratrace concentration level. It can be easily coupled with HPLC or CT and the limits of detection are comparable with ICP-MS detection which is much more expensive technique. Preliminary study of arsenosugors shows that Nori extract can be used as "standard" of PO_4-sug since it

contains about 95% of this compound and no matrix effect was observed during the HG.

Acknowledgments

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Developing of analytical micro concentrating systems for biomarker quantitative determination in exhaled air

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Keywords
biomarkers
breath air analysis
gas chromatography
micro concentrating systems
non-invasive diagnostics

Abstract
The aim of the work is to develop analytical micro concentrating systems and methods for concentrating trace aliphatic hydrocarbons from exhaled air samples. Analytical micro concentrating systems: chromato-desorption systems are demonstrated. These micro systems and methods allow to concentrate trace contaminants of aliphatic volatile organic compounds from exhaled air samples by solid phase microextraction technique. Three adsorbents (Chromaton N-AW-DMCS-15% PMS-1000, Al_2O_3, and MN-202) have been examined. The achieved concentration coefficients are also shown.

1. Introduction

Advanced non-invasive diagnostics method is breath air analysis [1–3]. It is based on determination of health functional disease selective biomarkers concentration in exhaled air. This analysis allows detecting diseases at early stages. About twenty components in breath air are health functional disease selective biomarkers [4]. Some of them are aliphatic hydrocarbons, e.g. lipid peroxidation biomarkers [5, 6].

Direct determination is limited by trace contaminant of biomarkers in exhaled air. For example, *n*-pentane (lipid peroxidation biomarker) concentration is 0–10 ppb, ethane (free radical caused destruction biomarker) concentration is 0–10 ppb, methane (gastrointestinal disease biomarker) concentration is 2–10 ppm [7]. Therefore, it is necessary to concentrate biomarkers from breath air samples.

The aim of this work is to develop analytical micro concentrating systems (chromato-desorption micro systems) and methods for concentrating trace aliphatic hydrocarbons from exhaled air samples.

To achieve this goal we have solved the following problems:

1. To make analytical micro concentrating systems;
2. To determine experimentally the possibility of concentrating with the use of developed micro systems;
3. To determine experimentally micro systems characteristics;
4. To determine experimentally the maximum concentration coefficients.

Relevance is driven by the need to develop an accurate rapid non-invasive diagnostics method which can be used on outpatient basis and reanimation basis. Developed chromato-desorption systems (CHDmS) have not been applied in practice previously.

2. Experimental

2.1 Reagents and chemicals

We have chosen three types of sorbents for micro systems Chromaton N-AW-DMCS-15% PMS-1000, Al_2O_3, and MN-202 and filled with them developed CHDmSs. We have used *n*-pentane as a target component to conduct experiment.

2.2 Instrumentation

Developed CHDmSs and methods allow to concentrate trace contaminants of aliphatic volatile organic compounds from exhaled air samples by solid phase microextraction technique. CHDmSs have been made from medical needles (inner diameter 0.5 mm) and filled with sorbents.

We have made analysis of sorbent surface microstructures using scanning election microscope Tescan VEGA. SEM magnification for Chromaton N-AW-DMCS-15% PMS-1000 has been 1.96 kx, for Al_2O_3 has been 3.00 k×, for MN-202 has been 2.00 k×.

Micro system capacitive characteristics have been conducted by means of passing *n*-pentane–air gas mixture through micro system. Concentrating of *n*-pentane from model gas mixtures with known analyte content has been conducted according to diagram shown at Fig. 1. Target component quantitative determi-

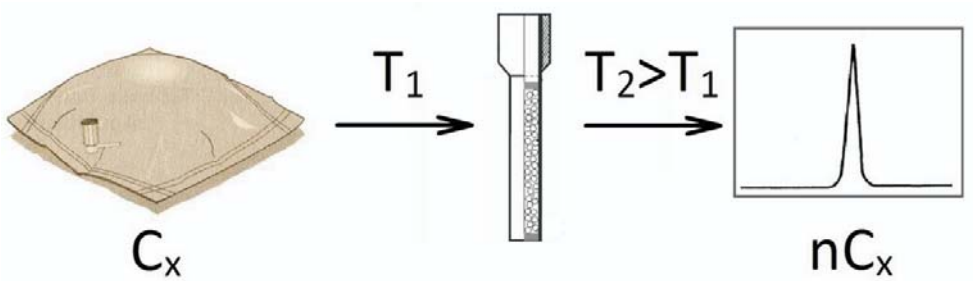
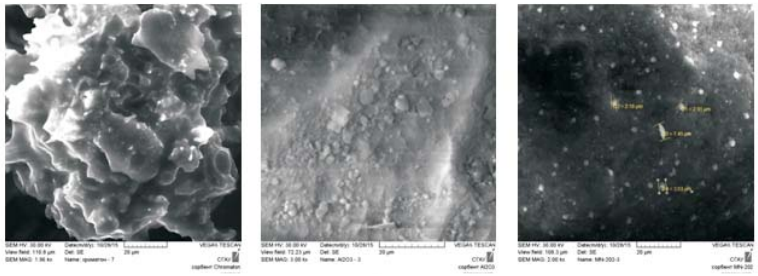


Fig. 1. Diagram of concentrating.



(a) (b) (c)

Fig. 2. Microphotograph of sorbent surfaces: (a) Chromaton N-AW-DMCS-15% PMS-1000, (b) Al_2O_3,(c) MN-202.

nation after concentrating has been made by using of gas chromatograph with flame ionization detector.

3. Results and discussion

Images of studied sorbent surface microstructures are shown at Fig. 2. It has been founded that Chromaton N-AW-DMCS due to 15% PMS-1000 modification has obtained highly extended surface structure and caused increasing of the specific surface and sorption properties (Fig. 2a). Al_2O_3 sorbent particles are small and have awkward geometric shape and also highly extended surface structure (Fig. 2b). Polymeric sorbent MN-202 surface (Fig. 2c) is characterized by macro- and micro- pores presence.

Micro system capacitive characteristics are shown at Fig. 3. According to CHDmS saturation curves Al_2O_3 sorbent saturation is achieved after passing 18 ml of model gas mixture, Chromaton N-AW-DMCS-15% PMS-1000 sorbent saturation is achieved after passing 10 ml and MN-202 sorbent saturation is achieved after passing 8 ml. Micro system sorptive capacities have been determined according to saturation curves (Table 1). The most effective sorbent is Al_2O_3 as it has the biggest sorptive capacity. Small sorptive capacity of polymeric sorbent MN-202 is attributed to system geometric parameters because of effective volume of sorbent is only 32% of total micro system volume.

Results of *n*-pentane concentrating from model mixtures by means of CHDmS are demonstrated in Table 2. CHDmSs make possible to concentrate target component by more than 50 times. The most effective micro system is CHDmS filled with Al_2O_3. Using this system we have achieved concentration coefficient $K = 54$. Such concentration coefficient is enough to determine biomarkers with ppm content in breath air, but bigger concentration coefficient value is desirable for determination of biomarkers with ppb content in breath air. And it can be acquired by means of CHDmS geometric parameters change.

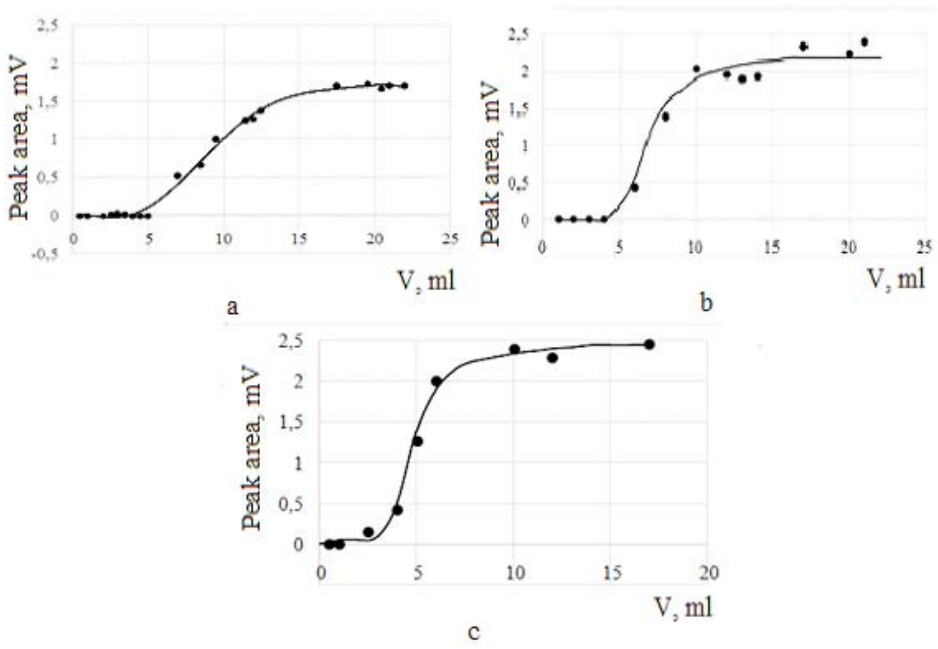


Fig. 3. Micro system capacitive characteristics: (a) Al_2O_3, (b) Chromaton N-AW-DMCS-15% PMS-1000, (c) MN-202.

Table 1
Micro system sorptive capacities (*n*-pentane mass [μg] sorbed on 1 mg of sorbent).

Sorbent	Capacity
Chromaton N-AW-DMCS-15% PMS-1000	5
Al_2O_3	20
MN-202	9

Table 2
Results of *n*-pentane concentrating: sample volume, desorption temperature, m_1 is *n*-pentane mass before concentrating, m_2 is *n*-pentane mass after concentrating, and *K* is concentration coefficient, $K = m_2/m_1$.

System	*V*, ml	*t*, °C	m_1, μg	m_2, μg	*K*
CHDmS (Al_2O_3)	20	150	0,1	5,4	54,0
	10	200	0,3	3,7	12,3
CHDmS (MN-202)	10	200	0,3	4,7	15,7
CHDmS (Chromaton N-AW-DMCS-15% PMS-1000)	10	200	1,9	4,8	2,5

4. Conclusions

Analytical micro concentrating systems and methods for concentrating trace aliphatic hydrocarbons from exhaled air samples have been developed. Characteristics of CHDmS filled with Chromaton N-AW-DMCS-15% PMS-1000, Al_2O_3, and MN-202 have been determined. It is reasonable to use CHDmS filled with Al_2O_3 to concentrate volatile organic compounds from breath air samples. Achieved concentration coefficient (K = 54) is enough to determine biomarkers with ppm content in breath air. Offered systems are portable and could be used in automatic analysis. CHDmS method meets green chemistry principles as it reduces dramatically chemical agent consumption. Developed micro systems can find application at medical institutions for disease diagnostics and health functional state damage risk identification.

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Chromato-desorption microsystems for the aliphatic hydrocarbons quantitative determination in conducting ecological and analytical control of the air indoor

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Keywords
ecological control
gas chromatography
microanalytical systems
air indoor quality

Abstract
The possibility of microanalytical systems complex use for the aliphatic hydrocarbons quantitative determination in ecological and analytical control of atmospheric residential and administrative buildings is shown in paper. The prototypes of chromato-desorption microsystems have been designed, experimentally studied and tested. It is injection type devices (25 mm × 0.5 mm). Chromato-desorption microsystems operational life is less than 8 cycles with standard error δ = 15% under conditions of discrete dosing. The offered method significant advantage is an ability of multiple-point calibration by changing the desorption temperature. The comparative accuracy of quantitative *n*-pentane determination showed a 15% advantage over the conventional methods. This was accomplished using the developed methods and devices.

1. Introduction

Life quality substantially depends on the ecological and hygienic housing characteristics. The World Health Organization experts emphasise that quality of internal air of various buildings and structures is more important for human health and well-being than the quality of the outdoor air [1]. According to international statistics, 30–70% of modern buildings have unrelated to the production and professional process problems with air pollution [2, 3]. The indoor air contains about 560 volatile organic compounds belonging to 32 groups of chemicals, the largest of which (44% of the organic compounds total amount) were hydrocarbons (saturated, unsaturated, aromatic, and cyclic) [4]. Aliphatic

hydrocarbons belong to fourn class of danger, but at high concentrations in the air, especially in enclosed spaces, substances are a toxicological hazard: they are characterized by excitation of the nervous system due to the additive effect of aliphatic compounds. Therefore, the content of substances even at trace level cause a significant harm to human health provides that permanent or regular presence in these areas. According to the World Health Organization, each year 4.3 million people die due to the influence of the indoor air pollution on their health [5]. Pentane was selected as the object of investigation, because of the similarity of physical and chemical properties to the volutale alkanes (C1–C4) and at the same time pentane exhibites the physical and chemical properties of the semi-volutale alkanes (C5–C8) [6].

The limiting factors, determining the accuracy and speed of measurement, are the calibration and sampling. The most important requirement for process calibration is to ensure the most accurate matching calibration conditions and subsequent analysis of the sample. The calibration gas chromatographs involve the use of calibration gas mixtures. When storing the volatile compounds of the calibration gas mixture occur analyte loss due to adsorption, so it is advisable to prepare a standard mixture directly in the analysis [7–9].

The aim of this work is development of methods and equipment for an adequate calibration and for quantitative analysis of the aliphatic hydrocarbons in indoor air.

2. Experimental

Chromatec Crystal 5000.1 has been used for the quantitative analysis of *n*-pentane by gas chromatography. The accuracy of quantitative analysis is limited by the sampling stage. Carrying calibration and sample preparation stages under identical conditions can reduce the error [10]. The analytical chromato-desorption microsystems (ChDmS) which can be used for obtaining calibration gas mixture and concentrating have been developed. The method is to saturate evenly with volatile organic compounds of the inert gas stream as it passes through ChDmS. This device is a tubular flow-through placing, filled with sorbent with a known amount of sorbed volatile organic compounds on it. In the study of these systems three types of sorbent have been used: MN-202, Al_2O_3, and Chromaton N-AW-DMCS coated with semivolatile liquid PMS-1000. Laws of gas chromatography-desorption method is described in the work of the adsorption equilibrium concentration method [11, 12].

Preparation of calibration gas mixtures was performed using the developed devices, which are ChDmS and allowing for the grading and concentration in adequate conditions. The principle of the method is the following: the inert gas stream is equilibrium saturated with volatile organic compounds when it passes through ChDmS which is a tubular flow-through container filled with sorbent which contains a known amount of volatile organic compounds (three kinds of sorbent used were: MN-202, Al_2O_3, and Chromaton N- AW-DMCS ICP-1000).

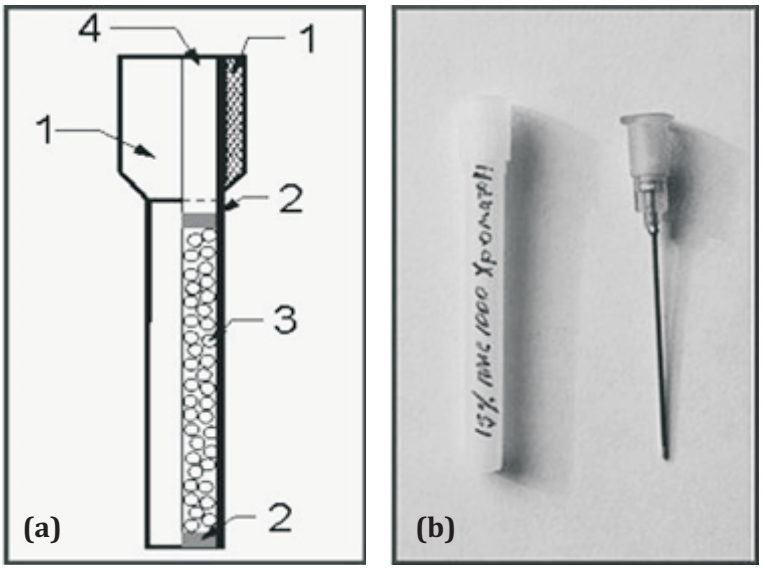

Fig. 1. (a) The schematic diagram of microCDS, and (b) photo of fabricated device: 1 – needle hook, 2 – plug, 3 – sorbent, 4 – internal channel of needle.

3. Results and discussion

Fig. 1 shows a schematic diagram ChDmS and a photo of the finished device. ChDmS represent a medical needle (40 mm length) with an inner diameter of 0.5 mm, filled with the sorbent (Chromaton N-AW-DMCS, MN-202, and Al_2O_3). Fig. 2 shows photographs of sorbent particles the microstructure surface analysis of the sorbent grains showed that there are expected anomalous manifestations of surface effects to the sorbent brand Chromaton N-AW-DMCS in the microsystems test configuration, which are not observed in the case of macro. This is due to the fact that Chromaton N-AW-DMCS with modification of 15% PMS acquires a strongly developed surface structure, which greatly increases its surface area, moreover sorbent particles are small and have irregular geometric form, whereby the packing density of ChDmS increases (Fig. 2a). The cumulative effect of these factors makes it possible to predict an increase in sorption capacity of the system, but there is low gas throughput in these systems. A similar effect of increasing the sorptive capacity of the system due to the large surface area and is shown to Al_2O_3 (Fig. 2b), the capacity reduction is not observed. The polymer sorbent MN-202 is characterized by the presence of the wall effect, characteristic of the configuration of the system under study ChDmS filled with sorbent of this type, due to the presence of the dead volume, which will result in reduced effective volume and as a consequence a reduction of the sorption capacity of the system.

It was established experimentally that ChDmS life of dispensing discrete gas mixture of at least 5 cycles with a standard deviation δ = 12-16% (Fig. 3).

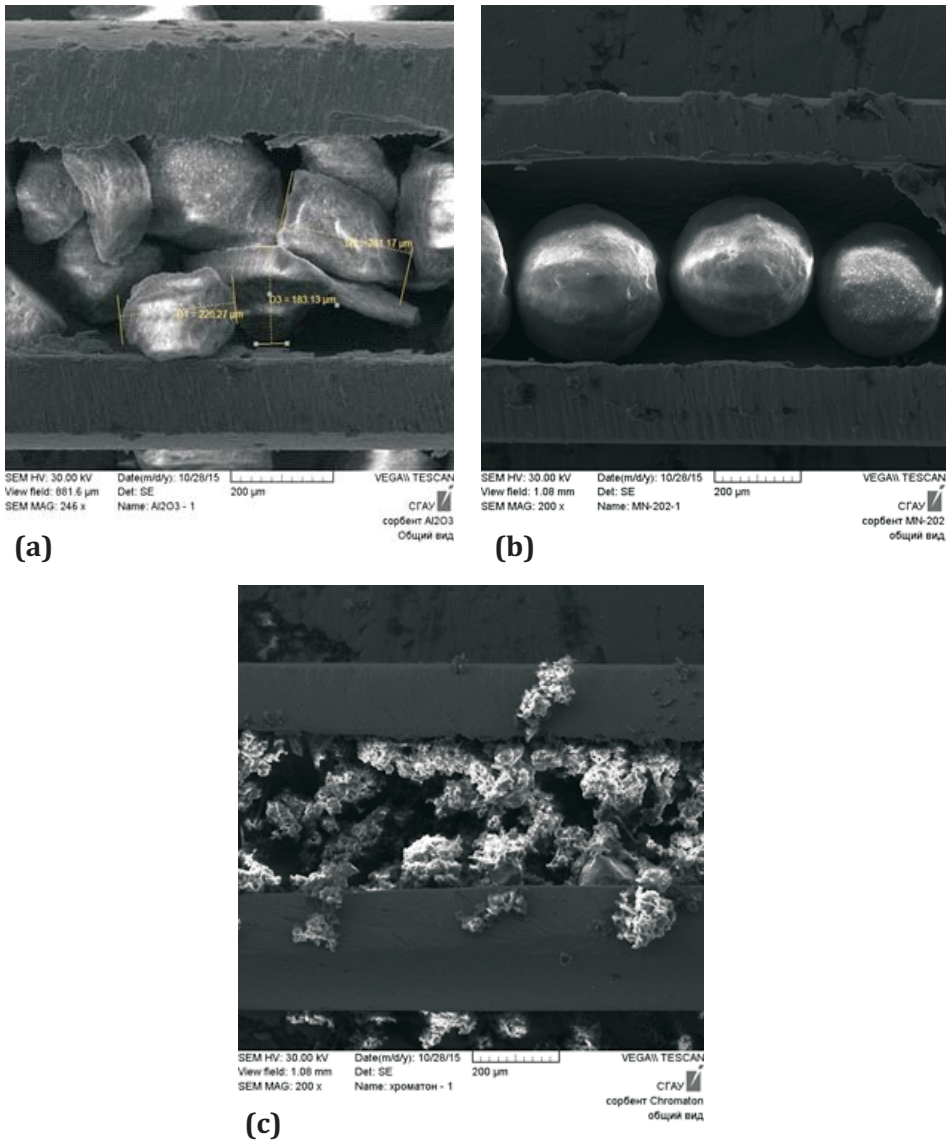

Fig. 2. The SEM image of sorbents: (a) photo of system cross section filled with Al_2O_3, (b) photo of system cross section filled with MN-202, (c) photo of system cross section filled with Chromaton N-AW-DMCS.

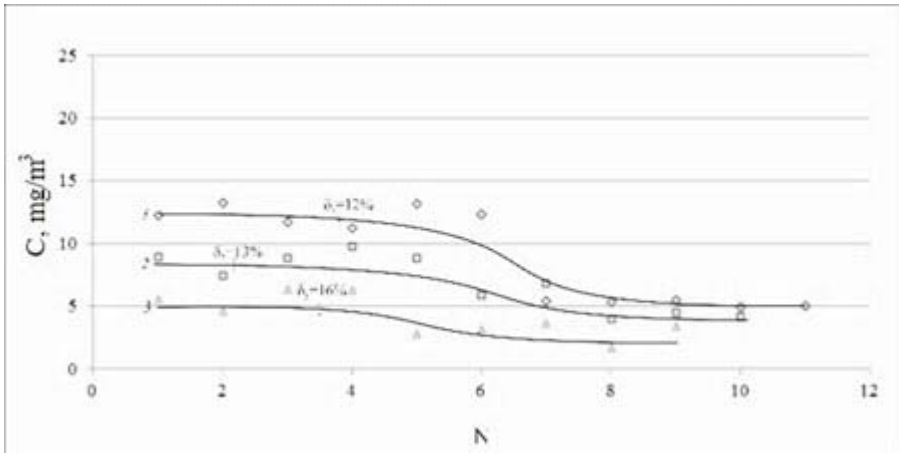

Fig. 3. *n*-pentane concentration in dependence on ChDmS filled Al_2O_3 sorbent usage period under conditions of discrete dosing. Desorption temperature: (1) 70 °C, (2) 100 °C, (3) 120 °C.

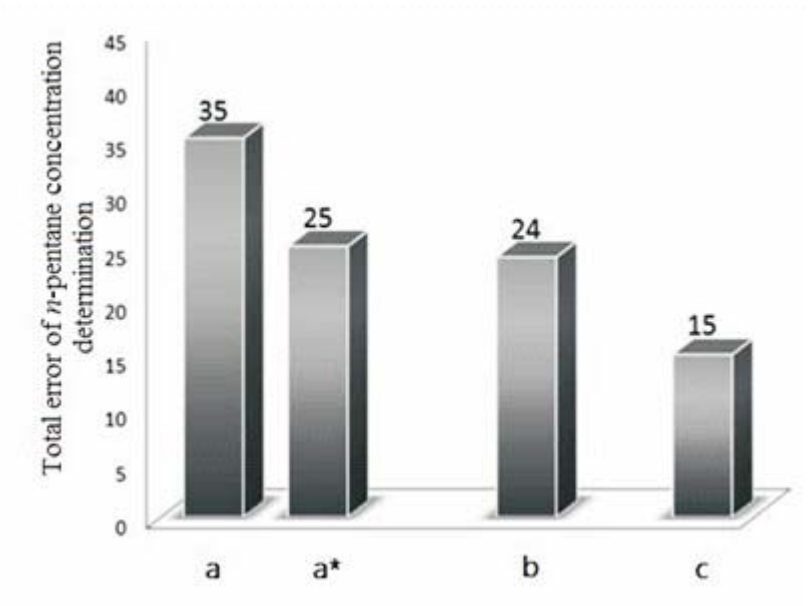

Fig. 4. Total error of *n*-pentane concentration determination used calibration gas mixtures diagram: (a) liquid calibration mixtures (with solvent background), (a*) liquid calibration mixtures (without including solvent background), (b) calibration gas mixtures prepared by static gravimetric method, (c) calibration gas mixtures prepared by discrete dynamic chromato-desorption method.

Table 1
Metrological characteristics analysis of convergence limit and reproducibility limit of the calibration gas mixtures preparation: *c* – analyte concentration in calibration mixture, *r* – convergence limit,

Method	*c*, mg/m^3	*r*, %	*R*, %	*K*
Static gravimetric method	10	12,23	17,61	0,91
	1	18,35	22,12	
Discrete ChDS method	10	12,10	15,65	0,92
	1	12,36	15,70	

An important advantage of the proposed method is the possibility of constructing a multi-point calibration by changing the desorption temperature.

Table 1 shows the results of convergence limit and reproducible preparation of calibration gas mixtures obtained in various ways. Convergence limit calculated for $n = 5$, where n is the number of measurements obtained at the same level (the same operator, under identical conditions); calculated reproducibility limit for $p = 3$, where p is the number of operators involved in the evaluation under identical conditions. As can be seen from the data on the characteristics of repeatability and convergence chromatography-stripping method of producing gas mixtures is not inferior to the well-known static gravimetric method for producing gas mixtures.

The standard methods of quantitative determination of *n*-pentane in air [10] suggest usage of liquid calibration solutions. Importantly, the use of liquid calibration solutions limit the calibration range by level of solvent purity, which in most cases has a trace of the analyte or other interfering trace that makes it impossible to directly determine the n-pentane at the desired level. To assess the adequacy of the calibration standard methods have been implemented: the grading on the liquid mixture and the gas mixture obtained in the static gravimetric method, as well as calibration with the ChDmS in a discrete implementation. Fig. 4 is a graph of the correlation between total error of measurement of the concentration of n-pentane and the applied calibration mixtures. As can be seen from the data the use of calibration mixtures obtained by chromatography-desorption method improves the accuracy of the analysis (ie, to reduce the total error analysis) by 10–13%.

4. Conclusions

Chromato-desorption microsystems have been developed. Calibration of analytical equipment and sample preparation were carried out under identical conditions by usage of the ChDmS. It has been found that the use of calibration mixtures obtained by chromato-desorption method improves the accuracy of analysis (i.e., reduce the total error of analysis) by 10–13%. The findings suggest that the developed ChDmS are suitable for the implementation of environmental

monitoring rindoor air. Moreover, the use of analytical microconcentrating systems complies with the «green» chemistry requirements due to significant reduction of resources consumption and chemical reagent consumption. It excludes completely the use of organic solvents if using thermal desorption.

Acknowledgments

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Expansion of the new method "Ethanol as Internal Standard" for direct determination of volatile compounds in the spirit products

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alcohol-containing products
ethanol
internal standard
validation
volatile compounds

Abstract
The method "Ethanol as Internal Standard" for direct correct determination volatile compounds in alcohol production is proposed. To simplify the validation of the method in the testing laboratory practice, on-line calculator AlcoDrinks was developed and located on the Internet at http://inp.bsu.by/calculator/vcalc.html. In this paper it was theoretically well grounded and experimentally demonstrated the possibility to validate the proposed method on the base of experimental data obtained in the laboratory during the tests in accordance with the Commission Regulation (EC) No 2870/2000.

1. Introduction

In accordance with the Commission Regulation (EC) No 2870/2000 [1], the official methods of the Association of Official Analytical Chemists (AOAC) [2, 3] and the official methods of International Organisation of Vine and Wine (OIV) [4, 5] determination of volatile compounds in the alcohol production is performed on gas chromatographs. Analysis of the quantitative content of test compound is carried out by the internal standard method. To represent measured values of the concentrations of analyzed volatile compounds according to [1–5] in mg/l of anhydrous ethanol (absolute alcohol) one should perform an additional procedure for measuring the volume of ethanol content in the test sample. The procedure for determining the volumetric ethanol content limits the minimum test sample volume in several tens ml [1]. It should also be noted that when testing alcohol-containing products with a noticeable volume content of volatile compounds (more than 0.5% by volume), the use of international alcoholometric water-ethanol tables [6] does not provide the required accuracy in determining the volume ethanol content of 0.1% by volume, since data tables are created only for binary water-ethanol solutions.

2. Theoretical basis

The cited above problems are solved in the proposed method "Ethanol as Internal Standard" [7, 8]. The method [7] consists in using ethanol as an internal standard. The method eliminates the need to add any substance as internal standard in the alcohol-containing sample, because this sample already contains ethanol.

In accordance with the requirements of ISO/IEC 17025 [9], developed new method must be validated before the application in practice of the testing laboratory. To facilitate the implementation of the proposed method in the practice of such laboratories we have proposed an algorithm of its validation on the basis of experimental data obtained during routine tests of alcohol products, in accordance with international standards [1–5]. It is important to notice that no additional measurements more than indicated in [1] are required, because in this case the measured chromatogram of prepared in accordance with [1] standard solutions can be calculated in accordance with our direct determination method [7, 8] and with conventional method [1]. Calculated in both cases values approximation coefficients R^2 and values of relative bias ΔU of measured values of concentrations of volatile compounds must be checked for compliance with the requirements of paragraphs 8.4 and 10 [1].

2.1. Description of validation algorithm

Results of test of standard solution "C" are used to determine the values of relative response factors RRF, prepared according to paragraph 5.14.3. The test results of standard solutions "0.1", "0.5", "1.0", "2.0" are used to determine the approximation coefficients R^2, that characterize the detector response linearity over the operating range of concentrations of studied quantities of volatile compounds [1]. In the case of using ethanol as internal standard the values of RFF_i^{ethanol} coefficients of *i*-th test compound can be summarized as follows:

$$RFF_i^{\text{ethanol}} = \frac{A_{\text{ethanol}}^{\text{st}}}{A_{\text{i}}^{\text{st}}} \frac{C_i^{\text{st}}}{\rho} \tag{1}$$

where A_i^{ethanol} and A_i^{st} are the values of response, for example, areas under the peaks, of ethanol as measured standard solution "C" and *i*-th test compound, C_i^{st} is a concentration of *i*-th test compound in mg/l of absolute alcohol, ρ = 789300 mg/l is the ethanol density.

The value of concentration of *i*-th C_i^{st} in mg/l examined when using ethanol as internal standard is defined by the expression:

$$C_i = RFF_i^{\text{ethanol}} \frac{A_{\text{i}}}{A_{\text{ethanol}}} \rho \tag{2}$$

where A_i and A_{ethanol} are values of the areas under the peaks, of *i*-th compound and ethanol when measuring the test sample.

In the case of using 1-pentanol as an internal standard, values of the coefficients of *i*-th testing compound can be summarized as follows:

$$RFF_i^{\text{pentanol}} = \frac{A_{\text{pentanol}}^{\text{st}}}{A_{\text{i}}^{\text{st}}} \frac{C_i^{\text{st}}}{C_{\text{pentanol}}^{\text{st}}} \tag{3}$$

where A_i^{pentanol} and A_i^{st} are values response, for example, areas under the peaks, of *i*-th test compound and ethanol solution in the measurement standard solution "C", C_i^{st} is a concentration of the *i*-th compound of the test in mg/kg of the solution, and C_i^{pentanol} is a pentanol concentration in mg/kg of solution.

Concentration value C_i in mg/kg of *i*-th investigated compound using pentanol as internal standard is defined by the formula:

$$C_i = RFF_i^{\text{pentanol}} \frac{A_{\text{i}}}{A_{\text{pentanol}}} C_{\text{pentanol}} \tag{4}$$

where A_i and A_{pentanol} are values of the areas under the peaks of *i*-th compound and pentanol when measuring the test sample.

Presentation of concentration values of examined compounds C_i is the required in accordance with [1], using the pentanol as internal standard, dimension mg/l (absolute alcohol) is performed according to the formula:

$$C_i\,[\text{mg/l}] = \frac{C_i\,[\text{mg/kg}]\rho_{\text{test}}[\text{kg/l}]}{strength(v/v)} \tag{5}$$

where ρ_{test} is a density of the test sample in kg/l, *strength*(v/v) is a volumetric content of ethanol (strength) in the test sample, expressed as a dimensionless quantity.

In case of constructing the linear dependence, passing through zero, of the detector response with respect to concentration of the volatile compounds, the value of approximation coefficient R^2 is determined by the formula [10]:

$$R^2 = \frac{\sum_{(i)} (C_i^{st})^2}{\sum_{(i)} (C_i)^2} \tag{6}$$

where C_i^{st} and C_i are values of volatile compounds in the standard solution, defined in the preparation by gravimetric method and calculated from chromatographic data. Approximation coefficients R^2 are determined from experimental data of standard solutions "0.1", "0.5", "1.0", "2.0". Finally, the results obtained from different methods, their relative bias and repeatability compared with each other.

3. Experimental

All individual chemical compounds were purchased from Sigma-Fluka-Aldrich. Standard solutions were prepared gravimetrically by adding individual chemical

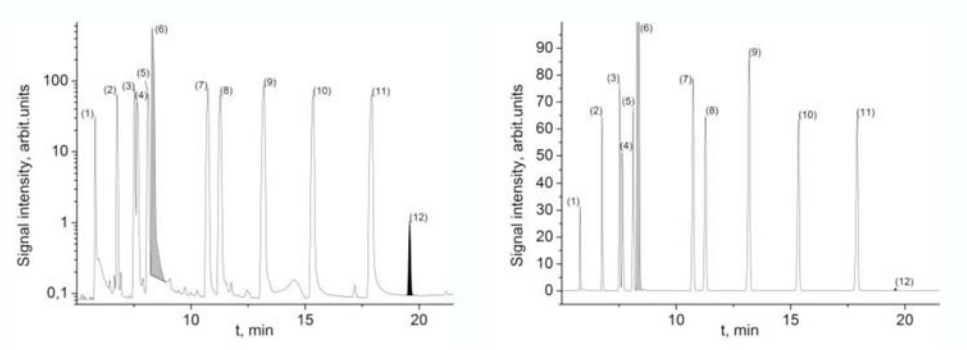

Fig. 1. Type of chromatograms of standard solution "A" with the following compounds: (1) acetaldehyde, (2) methylacetate, (3) ethylacetate, (4) methanol, (5) 2-propanol, (6) ethanol, (7) 2-butanol, (8) 1-pro-panol, (9) isobutanol, (10) n-butanol, (11) isoamilol, (12) 1-pentanol.

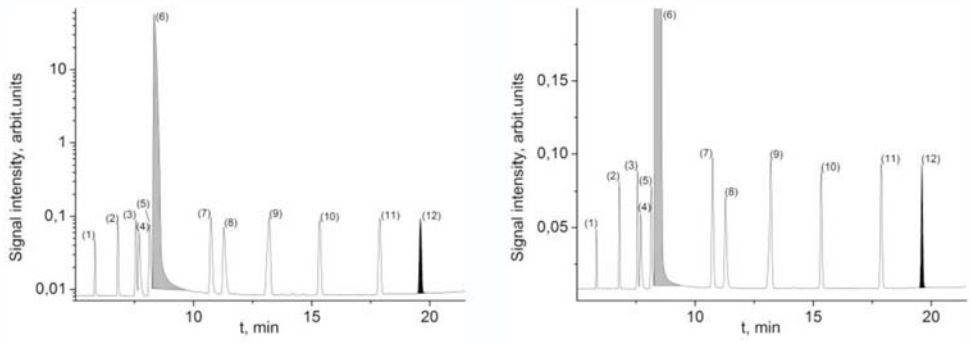

Fig. 2. Type of chromatograms of standard solution "C" with the following compounds: (1) acetaldehyde, (2) methylacetate, (3) ethylacetate, (4) methanol, (5) 2-propanol, (6) ethanol, (7) 2-butanol, (8) 1-pro-panol, (9) isobutanol, (10) n-butanol, (11) isoamilol, (12) 1-pentanol.

compounds in the ethanol-water solution with a volumetric content of 40% ethanol in accordance with [1]. Experiments were carried out in the Laboratory of Analytical Research of the Institute for Nuclear Problems of Belarusian State University. GC methodical parameters are presented in [7]. In order to validate the method in an extended with comparison [1] range of the studied concentrations of volatile compounds, standard solution of "A" has been tested as well as prepared standard solution "Min 1.0"with extremely low compound concentrations. The measured chromatograms of standard solutions "A" and "C" are presented in Fig. 1–2.

4. Results and discussion

Values of approximation coefficients R^2 are calculated from the experimental data obtained by method “Ethanol as an internal standard” when testing standard

solution "C" for all volatile compounds are greater than the value 0.999291. This is fully satisfies the requirements of paragraph 8.4 [1] At the same time, the value R^2 determined using traditional method of internal standard [1] exceed 0.999214. The values of relative bias ΔU calculated from experimental data of tests of standard solution "QC", for all compounds are not greater than 5.2%. At the same time, the magnitude of the relative bias determined using conventional internal standard method [1], reaches values of 9.1%.

The values of relative bias ΔU calculated from the results of tests of standard solutions "0.1", "0.5", "1.0" and "2.0" for all volatile compounds does not exceed 6.4%. At the same time, the value of the relative bias determined using conventional internal standard method [1] reaches values of 11.2%.

Analysis of experimental data obtained in test of standard solutions "A", "C", and "Min 1.0" indicates that the proposed method provides high metrological para-meters determining volatile compounds when changing the values of their concentration in the test sample from units of milligrams per liter of anhydrous alcohol in a standard solution "Min 1.0" till 83000 mg per liter of absolute alcohol in standard solution "A".

To demonstrate the high degree of simplicity of the method, on the one hand, and the high data reliability, let us consider results of experimental analysis of a certified reference material CRM LGC 5100 Whisky-Congeners. Fig. 3 presents the measured chromatogram in logarithmic and linear scale (at the top right). Error for methanol, 1-propanol, and isobutanol was less than 10%. The error for isoamilol is equal to 10.8%. For 1-butanol it was equal to 15.4% accuracy whereas

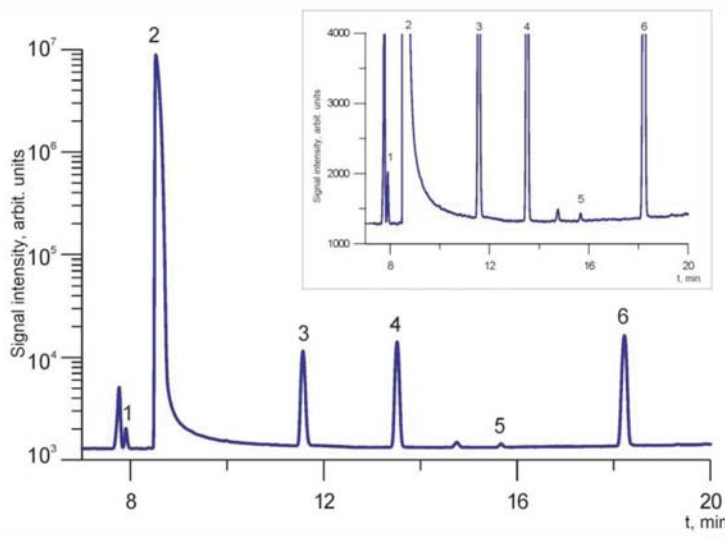

Fig. 3. Details of the chromatogram in logarithmic and linear scales (1) methanol, (2) ethanol, (3) 1-propanol, (4) isobutanol, (5) 1-butanol, (6) isoamilol.

according to certificate it is 22.9%. It follows that the proposed method gives satisfactory results at the level of the 16 competent testing laboratories participated in the research of this reference material CRM LGC 5100 Whisky-Congeners [11].

4. Conclusions

Method of direct determination of volatile compounds in alcohol products with the use of ethanol as an internal standard allows the direct determination of the concentrations of the volatile compounds in the required dimension mg/l of anhydrous ethanol in full compliance with international regulations [1–5]. Direct determination method allows simplify the whole test procedure, since in this case there is no need to perform a manual procedure for addition the internal standard substance in the prepared standard solutions and in test samples, too. Method provides possibility to correctly determine concentration values of the volatiles compounds in the test alcohol samples when the volume content of volatile compounds exceeds 0.2%. Method of direct determination can be validated in the test laboratory directly from the experimental data obtained in the tests of alcoholic products in accordance with [1]. No further measurements, more than indicated in [1], are required. It is sufficient to perform the following steps: the measured chromatogram of standard solutions prepared in accordance with [1] should be calculated by the method of direct determination using ethanol as internal standard [7, 8] and by conventional method of internal standards [1]. Calculated in both cases values of approximation coefficients R^2 and values of relative bias (trueness) of measured values of concentrations of volatile compounds can be checked for compliance with the requirements of paragraphs 8.4 and 10 [1].

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Age determination of fingerprints by GC-MS

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squalene

Abstract
The aim of this work was to develop an extraction technique for fatty acids and squalene from fingerprints. To increase the sensitivity of the mass spectrometric detector, samples were analysed in SIM mode. Newly developed method allows monitoring the composition of organic compounds in a single fingerprint extracted from glass surface, and allows the analysis of changes in the composition of organic substances exposed to daylight for several days. From the arising changes we are able to determine the age of the fingerprint.

1. Introduction

Fingerprints found their application mainly in forensic sciences but despite the high public interest and large number of studies, there are many insufficiently explored areas such as age determination of latent fingerprints which still need extensive research. Fingerprints have a complex composition. They contain substances from the eccrine (sweat) glands including water, inorganic salts (Na^+, K^+, NH_3, NH_4^+ , Ca^{2+}, Mg^{2+}), uric acid, vitamins, creatinine, chlorides, polypeptides, amino acids, urea and other components [1]. Fingermark residue contains sebaceous compounds as glycerides, cholesterol, cholesterol esters, waxes, wax esters and squalene [2]. Hands and feet are free from sebaceous glands, it means, that sebum is transferred into fingerprints after contact with parts of the body, where this secret is produced (face, hair, shoulders, chest) [1]. Furthermore, fingerprints contain components from the epidermis, metabolites of drugs, external contaminants such as nicotine, remains of food and cosmetics and other substances to which the donor is exposed during the day [4–6]. It was also observed that the composition of fingerprints of a same donor (intra-variability) and of different donors (inter-variability) is highly variable [3].

The composition of fingerprints can be changed through various chemical, physical and biological processes, including degradation, drying, evaporation,

metabolism, migration, oxidation and polymerization [1]. Squalene, the most abundant and most often used compound for fingerprint aging determination, undergoes relatively rapid degradation, mainly if exposed to light, it can be decomposed/ oxidized in two weeks [1]. Because each individual has different amounts of squalene, it is not enough to monitor the kinetics of decomposition of this substance and the amounts of their oxidation products in fingerprint for age determination.

The aim of this work was to monitor the amount of selected stable saturated fatty acids (palmitic acid, stearic acid) in the samples, which we have compared with squalene in order to determine the age of a fingerprint extracted from a glass surface.

2. Experimental

2.1 Reagents and chemicals

Chloroform was bought from J.T.Baker (Deventer, Netherland). Dichloromethane, hexane and sodium methoxide were purchased from Merck. Squalene, oxalic acid and diethyl ether were ordered from Sigma-Aldrich. Methanol was bought from Fisher Scientific.

2.2 Extraction of fingerprints

Fingerprint samples were collected from the volunteers with a condition that they did not wash hands for at least half an hour before sampling. Samples were obtained by pressing the fingertip against a watch glass for 10 seconds. The samples were extracted into 500 µl of hexane, transesterified by the procedure listed below and the resulting solution were taken up in 2 ml vials.

2.3 Derivatization

The methyl esters of fatty acids were prepared using basic transesterification by adding 50 ml of a 0.5 M solution of sodium methoxide in dry methanol to the fingerprint samples. Samples were mixed and reacted at 40 °C for 15 minutes. In the next step 50 ml of oxalic acid (0.5 g in 15 ml of diethyl ether) was added to the solution, it was mixed thoroughly and centrifuged at 2000 rpm for 3 minutes to settle the precipitated sodium oxalate.

2.4 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 and SIM mode. Chromatographic separation was carried out on a 30 m × 0.25 mm × 0.25 μm DB-5 capillary column. Samples with injection volume of 1 μl were injected in splitless mode with splitless time set to 2 minutes. Helium was used as a carrier gas with constant pressure at the injector 102 kPa. Injector temperature was set to 280 °C. The oven temperature was set to 60 °C for 1 minute and gradually increased to 310 °C at a rate of 15 °C min^{-1} and was held for 3 minutes.

3. Results and discussion

Fig. 1 shows a chromatogram of an extracted fingerprint after basic transesterification. The analysis was realized in SCAN mode to identify individual substances contained in the fingerprint. From Fig. 1 is obvious that the major compounds contained in the extract are squalene and methyl esters of palmitoleic, palmitic and oleic acids. These fatty acid methyl esters are exclusively from triglycerides and therefore are not affected by the presence of free fatty acids in the fingerprints which are more mobile and less stable than the acids present in triglycerides.

To determine the age of the fingerprint we have selected the ratio of squalene to stearic acid present in triglycerides assuming that the content of stearic acid does

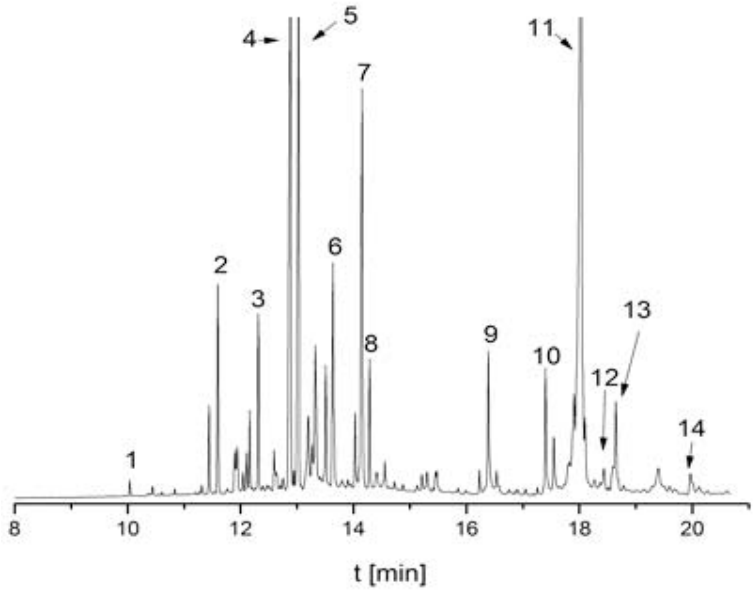

Fig. 1. GC-MS SCAN chromatogram of transesterified fingerprint: (1) lauric acid (C_{12}) methyl ester, (2) methyl myristate (C_{14}), (3) pentadecanoic acid methyl ester, (4) palmitoleic acid methyl ester, (5) palmitic acid methyl ester, (6) isopropyl palmitate, (7) oleic acid methyl ester, (8) stearic acid methyl ester, (9) behenic acid (C_{22}) methyl ester, (10) lignoceric acid (C_{24}) methyl ester, (11) squalene, (12) cerotic acid (C_{26}) methyl ester, 13-cholesta-3,5-diene, 14-cholest-5-ene-3-ol.

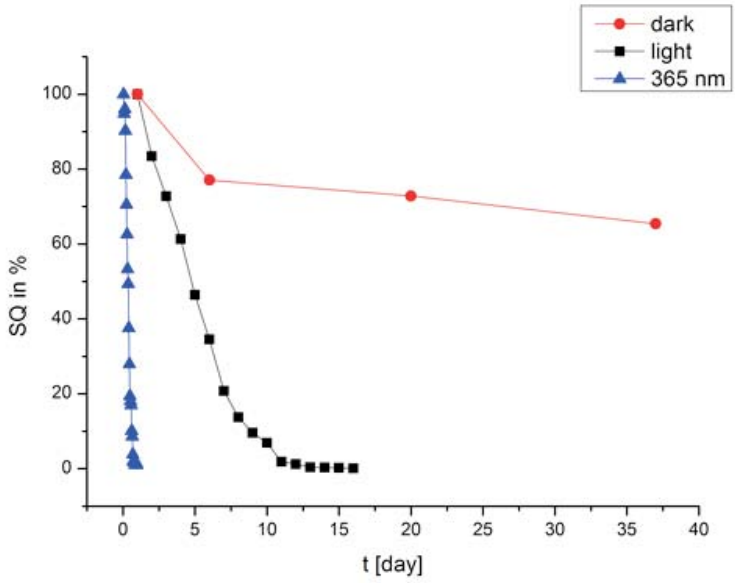

Fig. 2. Impact of radiation on concentration of squalene.

not change during the studied period while the content of squalene decreases by the influence of light and atmospheric oxygen. Fig. 2 shows the curve of decreasing squalene content in the presence of atmospheric oxygen when exposed to light of different wavelengths. The graph shows the rate of degradation kinetics of squalene under various conditions as light, dark conditions and under 365 nm. From the picture is evident, that the decomposition of squalene is fastest when irradiated with 365 nm, after one day it was not detectable from the samples because its concentration was below the detection limit. In dark conditions squalene is oxidized very slowly by atmospheric oxygen, but if exposed to light, it can be decomposed in two weeks.

Fig. 3 presents the ratio of squalene to stearic acid from triglycerides in a fingerprint under the influence of daylight. As can be seen, the ratio of squalene/stearic acid is decreasing while the content of stearic acid did not change in the fingerprint. Changes in this ratio were caused by the oxidation of squalene as the rate of oxidation is significantly influenced by the intensity of light. For the unambiguous fingerprint age determination it is also necessary to know the light conditions. Only the age of two different fingerprints on the same repository (e.g. glass, plastic) can be determined with certainty.

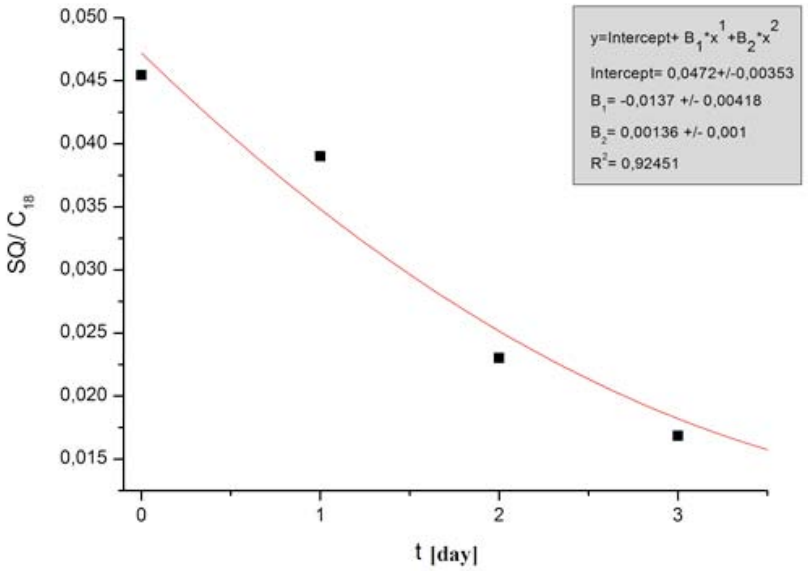

Fig. 3. Ratio of squalene to stearic acid depending on the action of daylight.

4. Conclusions

The aim of this work was to develop new extraction methods for squalene and fatty acids from fingerprints. As extracting agents for fingerprints we chose dichloromethane because of its good extraction capacity and rapid evaporation from the samples. For the treatment of extracted fingerprint samples we have chosen alkaline transesterification with 0.5M sodium methoxide. After transesterification of fingerprints we have increased the concentration of the samples and analyzed by a newly developed method with improved sensitivity of MS detector in SIM mode. In this work we have described the kinetics of squalene oxidation under different experimental conditions (dark, light and under 365 nm lamp). In the next step we have analyzed fresh fingerprints extracted from the glass surface and fingerprints exposed to daylight. By using preconcentration and a modified method we increased the sensitivity of the analysis so that we could analyze a fingerprint. Using this newly developed method we were able to detect squalene in fresh fingerprint samples and in samples exposed to daylight for 1, 2, and 3 days. From the obtained squalene/fatty acid ratio we are able to define and/or determine the duration of exposure to daylight of the fingerprint.

Acknowledgments

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The analysis of non-polar compounds in cigarette ashes by GC-MS

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Keywords
ash
burned cigarette
cigarette
GC-MS

Abstract
The work deals with determination characteristic substances in ash with goal to identify kind of ash. It is necessary nonvolatile substances before analysis to be appropriately adjusted. The method of the adjusting that we chose is silanization with HMDS and BSTFA. By comparing chromatographs of three commonly available brands of cigarettes in SCAN and SIM mode, for every single brand, we chose characteristic organic substances. By substances that we chose and through the method we can reliably differ three kinds of ashes of different brands.

1. Introduction

Many studies deal with determination of heavy metals and salts in ash and fly ash. Information about organic substances in ash are still very little. Among the researched belong, e.g., polychlorinated dibenzodioxins and polychlorinated dibenzofurans in different burnt rests of waste [1]. Except of these substances were identified also polycyclic aromatic carbohydrates [2], aromatic carboxyl acides [3, 4], and polychloric bifenyls [5]. For determination of these substances are used extractive techniques in connection with chromatographic methods as well. Mostly we use Soxhlet's extraction [6], supercritical fluid extraction, and simple extraction [7].

Only few of studies deal with analysis of polar substances in ash. Among these analyses belong determination of routine in tobacco, filters and in ash from different kinds of cigarettes [8]. Analysis of ash from tobacco is specific. From 3800 chemical substances only little part of them can preserve after burning their original structure. The substances that were identified in tobacco, were not found in ash. Among this kinds of studies belong, e.g., determination of routine or maleic hydrazine [9, 10].

The aim of our study was to determine to work out new method for direct silanization of polar substances in burnt cigarette ash with goal to determine source of ash in different kinds of cigarettes.

2. Experimental

2.1 Reagents and chemicals

1,1,1,3,3,3-hexametyldisilazane (HMDS) and acetonitrile were bought from Merck. *N,O*-bis (trimetylsilyl) trifluoroacetamide (BSTFA) and trifluoroacetic acid (TFA) were bought from Fluorochem. Tobacco products of brands such as Marlboro Touch (Phillip Morris), L&M Red (Phillip Morris), Davidoff Gold (Imperial Tobacco) were bought in a local shop.

2.2 Derivatization procedure

An amount of 50 mg of cigarette ash was flooded with 300 µl HMDS, 300 µl ACN and 2 µl TFA. The mixture was heated (50 °C) and then shaken in shaker. After 30 minutes, was in the mixture added 300 µl of BSTFA, it was heated (80 °C) and consequently shaken for 30 minutes. The mixture was centrifuged and the supernatant was collected.

2.3 Instrumentation

The ingredients was separated by GC-MS (Agilent Technologies) on column DB-5 MS, 30 m × 0.25 mm × 0.25 µm (Agilent Technologies). The injected volume of sample was 1 µl into the injector operating in splitless mode (2 min). 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 held for 13 minutes.

3. Results and discussion

Fig. 1 depicts chromatogram of tobacco and tobacco ash after silanization with HMDS and BSTFA. Chromatographic record from tobacco is formed by big amount of nonpolar as well as polar components. Identification of major components was confirmed by NIST library and it is in compliance with substances published till now. It is obvious, that in ash are not any substances from original tobacco on level of detective limit of method in SCAN mode. There was presence of new substances in trace concentration too.

According to measured weighable spectres of these substances, that we got as result of direct silanization of ash, we chose XY ions, that we were using during

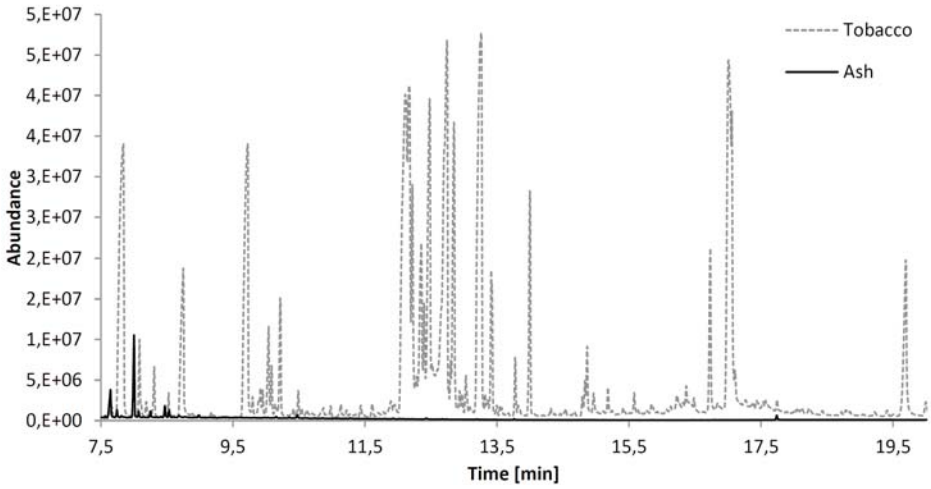

Fig. 1. GC-MS SCAN chromatographic records like finger print tobacco and burnt ash.

next measurements in SIM mode. These chosen substances have high selectivity to determine source of ash. By comparing chromatographic records from ashes of different kinds of cigarettes we determined XY substances that characterize ash from every single kind of cigarette. Fig. 2 depicts chromatographic records of ash from three different sources of cigarettes (Mars, L&M, and Davidoff). It is obvious that to determine source of ash is clearly possible. Next substances suitable for identification of source of ash are listed in Table 1. In the table are determined XY substances, that characterize ash from different kinds of cigarettes. For every substance is determined retention time, *m/z,* and its relative contain in different ashes. According the table is obvious that the greatest amount of characteristic substances were in ash from cigarette of brand Mars.

4. Conclusions

The newly method for direct silanization of nonvolatile substances developed by us in burnt cigarette ash is two-step silanization with HMDS a BSTFA. Based on GC-MS analyses we confirmed, that composition of ash depends on type of material that we burn. By newly developed method we were able to watch changes in ash from three different kinds of cigarettes. For every kind of ash were chosen few of characteristic substances, while for ash from L&M there were 4 substances, 6 substances for Davidoff, and 17 substances for Mars.

Acknowledgments

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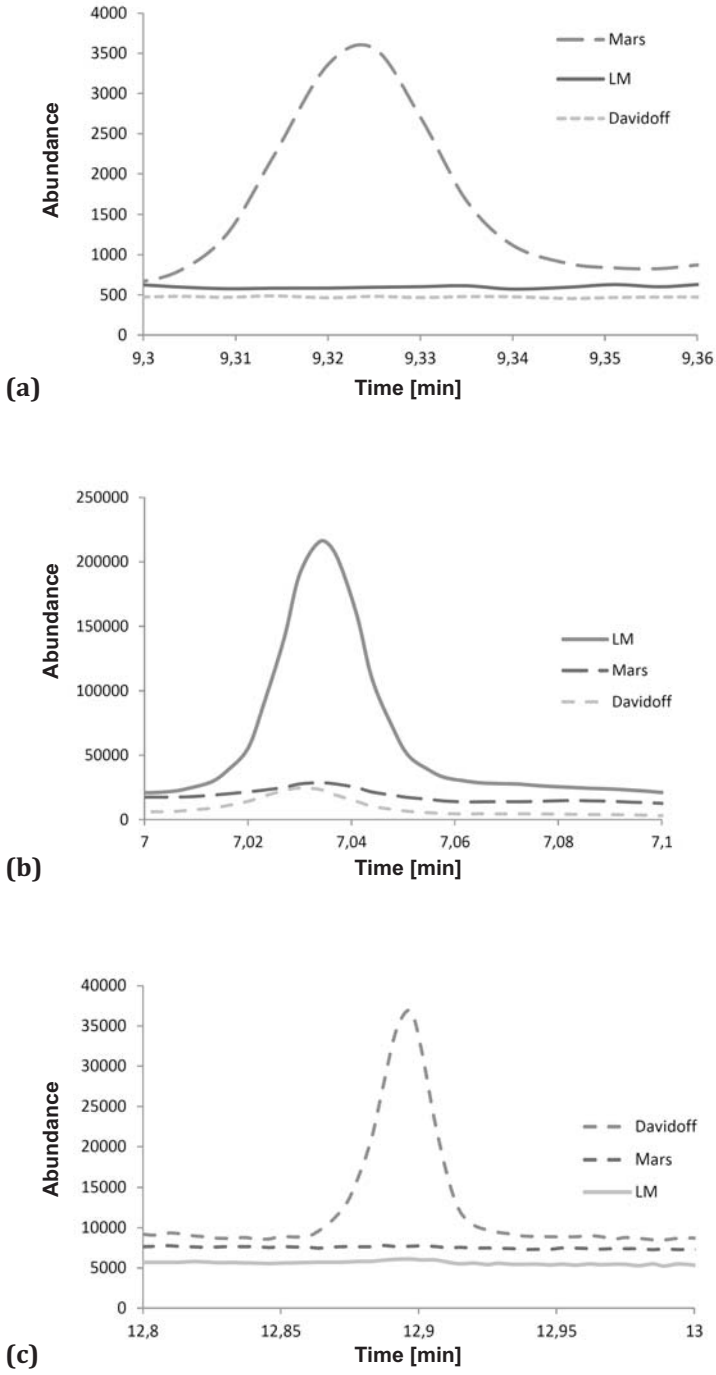

Fig. 2. Chromatographic record GC-MS SIM mode for (a) *m/z* = 73, (b) *m/z* = 104, (c) *m/z* = 147 for cigarette ash of tested brands.

Table 1
Selected substances in cigarette ash of tested brands (retention time, *m/z*, and source)

Number	t_r, min	*m/z*	Source		
			L&M	Mars	Davidoff
1	6.5	41, 77, 215, 232	100	–	–
2	7.0	77, 104	100	–	–
3	7.8	147, 233	–	100	–
4	7.9	137, 147	100	–	–
5	9.3	73	–	100	–
6	10.1	175 ,309	100	–	–
7	11.9	175, 217, 309	–	100	–
8	12.2	217	–	100	–
9	12.5	217	–	100	–
10	12.5	147	–	–	100
11	12.8	175, 217	–	100	–
12	12.9	147	–	100	–
13	13.1	121	–	100	–
14	13.6	217	–	100	–
15	13.6	147	–	–	100
16	13.8	217	–	100	–
17	14.5	217	–	100	–
18	15.3	73, 75	–	100	–
19	16.8	281, 437	–	100	–
20	16.9	217, 281, 361	–	100	–
21	16.9	129, 218	–	–	100
22	17.1	217	–	–	100
23	17.5	129, 218	–	–	100
24	17.5	217, 361, 437	–	100	–
25	18.1	217, 361	–	100	–
26	18.3	217, 361	–	100	–
27	18.4	217, 281	–	–	100

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The difficulties in polybrominated diphenyl ethers (PBDEs) identification by GC-EI-MS technique

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Keywords
debromization of dekaBDE
GC-EI-MS
PBDEs
selection of optimum GC conditions

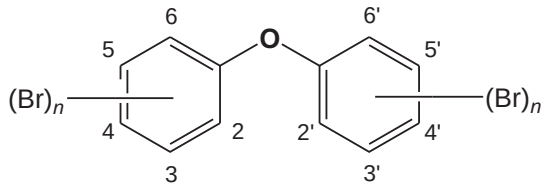
Abstract
Awareness about the harmful effects of brominated flame retardants on living organisms increases mainly due to link their presence in the human environment with health disorders. Therefore is a need to conduct research aimed at content control of these chemicals in the environment. Recent improvements in injection techniques and mass spectrometer ionization methods have led to a variety of options to determine PBDEs in environmental samples. Some difficulties in qualitative and quantitative analysis still make dekaBDE congener (BDE-209). Modeling studies aimed at selecting the optimal conditions for the separation and identification of selected PBDEs patterns have been carried out. The results are an introduction to the study of real environmental samples. Using technique was gas chromatography with electron ionization-mass spectrometry.

1. Introduction

Polybrominated diphenyl ethers (PBDEs) are highly effective flame retardants, introduced in middle of 1970s. It was then that PBDEs were commonly added to fabrics and synthetic materials (e.g., TV casings, wire insulations, computers, and carpets) during the manufacturing process [1]. Despite advantages of using brominated flame retardants, many PBDEs have been banned in production of consumer products in European Union and USA. The reason for such decision has become scientifically proven phenomenon of PBDEs bioaccumulation in the adipose tissue of living organisms and high persistence in the environment of this compounds [2].

The data on the toxicity of PBDEs are limited. Their harmful impact on human health is mainly related to the possibility of causing disorders of thyroid hormones production. Derivatives of PBDEs structurally are similar to thyroid hormones: diiodothyronine, triiodothyronine and thyroxine (T2, T3, and T4) and can be combined with hormone receptors present in the human body [3].

Table 1
IUPAC names, numbering system, and characteristic ions of the studied polybrominated diphenyl ethers.



IUPAC name	IUPAC number	Characteristic ions
2,4,4'-tribromodiphenyl ether	BDE-28	246, 248, 406, 408
2,2',4,4'-tetrabromodiphenyl ether	BDE-47	326, 328, 484, 486
2,2',4,4',5-pentabromodiphenyl ether	BDE-99	404, 406, 564, 566
2,2',4,4',6-pentabromodiphenyl ether	BDE-100	404, 406, 564, 566
2,2',4,4',5,5'-heksabromodiphenyl ether	BDE-153	482, 484, 642, 644
2,2',4,4',5,6'-heksabromodiphenyl ether	BDE-154	482, 484, 642, 644
2,2',3,4,4',5',6-heptabromodiphenyl ether	BDE-183	562, 564, 722, 724
2,2',3,3',4,4',5,6,6'-nonabromodiphenyl ether	BDE-207	720, 722, 880, 882
Decabromodiphenyl ether	BDE-209	400, 401, 797, 799

In addition, PBDEs are suspected obesogens – dietary, pharmaceutical, and industrial compounds that may alter metabolic processes and predispose some people to gain weight [4]. Exposure to PBDEs (before and after birth) can also cause problems with brain development [5]. Harmfulness of PBDEs shows the need to monitor their presence in the environment.

According to the literature, 209 congeners are classified into the group of PBDEs compounds. Chemical structure of selected PBDEs congeners with their characteristic ions, usually monitored with using GC-EI-MS technique, are presented in Table 1 [1, 6].

One of the greatest challenges to measuring PBDEs in environmental samples is developing methods to identification and accurately determination of decaBDE (BDE-209). This congener is not stable at high temperatures in the GC injector and GC column there occurs debromization. Moreover, BDE-209 is sensitive to degradation by UV light. In the MS source BDE-209 behaves differently than lower-brominated compounds [7]. In addition, BDE-209 may easily adsorb to small dust particles in the laboratory conditions, which may result in sample pollution. Thermal degradation BDE-209 can be avoided using a short GC column (e.g., 15 m) and short injector residence time (pulsed splitless injection) or on-column injection [8]. In other hand, lower-brominated congeners need for separation to use sufficiently long GC column. For example BDE-154 usually co-elutes from most gas chromatographic columns with 2,2',4,4',5,5'-hexabromobiphenyl (PBB-153), which is present in many environmental samples. Thus, in order to accurately quantification of BDE-209 and BDE-154 in environmental

samples is advantageous to perform two separate GC analyses under different operation conditions [6]. The present work describes results of research aimed at selection of optimal conditions for PBDEs separation and identification.

2. Experimental

2.1.Reagents and chemicals

The standard solutions of individual congeners: BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154, BDE-183 in isooctane, BDE-209 in isooctane:toluene 9:1 (concentration of each standard was 50 μg/mL) were purchased from Accu-Standard, USA. Internal standard solution of decachlorobiphenyl (PCB-209) was purchased from Sigma-Aldrich. Isooctane to preparing of analyzed mixtures was obtained from Merck.

2.2.Instrumentation

GC-EI-MS analyses of PBDEs were carried out on an Agilent 5977A mass spectrometer with quadrupole mass analyzer connected to an Agillent 7890A GC system equipped with an Agilent 7693 autosampler. The GC injection port was configured for 1 μl pulsed splitless injections. Gas chromatographic separation prior to MS was performed using a HP-5MS 30 m × 0.25 mm × 0.25 μm (J&W, USA). Helium was used as carrier gas. The GC parameters used to optimization of PBDEs separation conditions are given in Table 2. Ionization was performed by electron ionization (EI). The source temperature was 250 °C. The mass spectrometer was operated both in SCAN and SIM mode depending on the optimization parameters of the instrument.

Table 2
The GC parameters used to optimization of PBDEs separation conditions.

Parameter	First GC conditions	Second GC conditions
Injector temperature	290 °C	300 °C
Injection system	Pulsed splitless (50 psi, 4 min)	Pulsed splitles (25 psi, 4 min)
Flow rate	1 ml/min	2 ml/min
Oven temperature	90 °C for 1 min, 20 °C/min to 200 °C for 1 min, 4 °C/min to 290 °C, and 290 °C for 35 min	90 °C for 1 min, 30 °C/min to 300 °C, and 300 °C for 40 min
Total run time	70 min	48 min

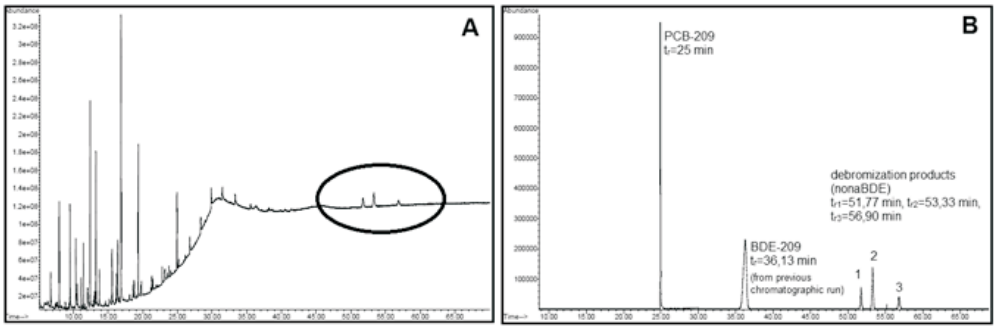


Fig. 1. The chromatograms of BDE-209 under first GC conditions: (a) SCAN mode, (b) SIM mode.

3. Results and discussion

For GC-EI-MS analyses, the identification was achieved by the retention time and maximal four characteristic *m/z* ratios (listed in Table 1). Analysis of PBDEs mixture under first GC condition (1 μg/ml of 7 congeners, 10 μg/ml of BDE-209, and with addition of 1 μg/ml PCB-209 as internal standard) showed that BDE-209 congener is not visible or visible in different retention time while repetitions of injections. Other congeners were visible on the chromatogram and good separated. This phenomenon was suggested the possibility of BDE-209 deposition in dosing system.

After liner replacement it was decided to dose only BDE-209 sample (50 μg/ml)spiked with internal standard. Results are shown in Fig. 1. Analysis in SCAN mode showed the presence of three peaks from which each have a similar mass spectrum. To increase the sensitivity, the SIM mode was used. This has highlighted that it were the products of BDE-209 debromization and BDE-209 is eluted before them. This fact pointed to mismatched conditions of the chromatographic analysis. It was concluded by this temperature and flow rate, the analysis time should be extended. Every time the data for BDE-209 was lost and he was eluted in the next chromatographic run.

After the conditions change (second GC conditions) BDE-209 sample (20 μg/ml should be sufficient to identify) with internal standard was dosed in SIM mode. Other temperature terms and a higher flow rate of the carrier gas caused a significant reduction of the analysis time required for the BDE-209 identification. Enlargement of chromatogram scale showed the presence of debromization products (Fig. 2). Analysis of PBDEs mixture showed that in new GC conditions analytes are good separated. NonaBDE are also present (Fig. 3).

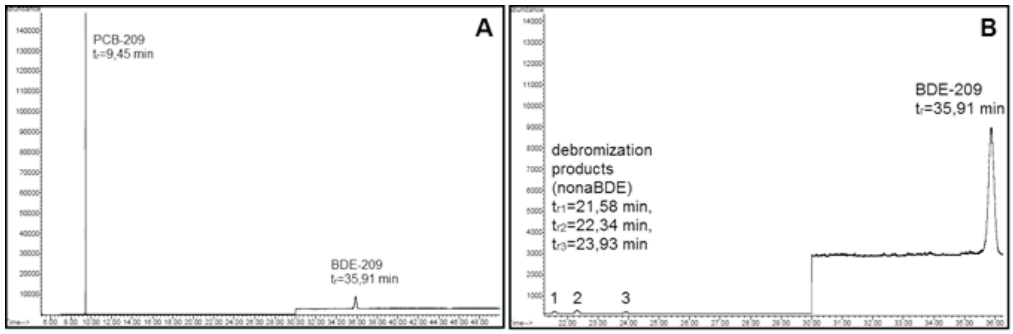


Fig. 2. Chromatograms of BDE-209 under second GC conditions: (a) SIM mode, (b) SIM mode, enlarged scale.

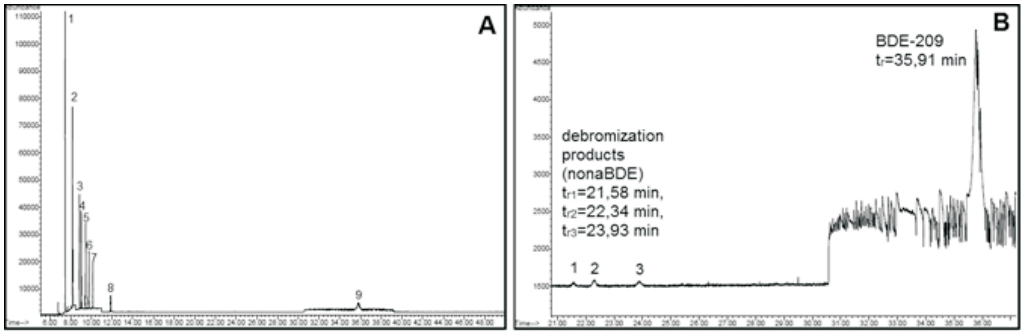


Fig. 3. Chromatograms of PBDEs mixture under second GC conditions**:** (a) SIM mode: (1) BDE-28, (2) BDE-47, (3) BDE-100, (4) BDE-99, (5) PCB-209, (6) BDE-154, (7) BDE-153, (8) BDE-183, (9) BDE-209; (b) SIM mode, enlarged scale

4. Conclusions

Degradation of PBDEs, particularly BDE-209, can occur on the GC column. The presence of hump, rising baseline or additional peaks before BDE-209 proves about degradation during injection, separation or in case of real samples also during extraction and clean-up. For this reason the choice of retention gap should always have an effect on the degradation of BDE-209 during analysis. BDE-209 can be measured with the other PBDEs, but will give a smaller and broader peak. Because the retention time is long, the determination of BDE-209 should be done separately, using thinner films (0.1 μm) and/or a shorter column, both of which should be help to improve the detection of BDE-209.

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Determination of rhenium complexes with 1,2-dihydroxybenzene and 1,2,3-trihydroxybenzene by CZE compared with HPLC-UV/MS

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Keywords
antitumor agent
coordination chemistry
CZE
HPLC
rhenium compounds

Abstract
Two analytical methods, HPLC and CZE, have been successfully used for determination and separation of reaction mixtures containing rhenium complexes, a potentially important group in medicine, prepared by a reaction of tetrabutylammonium tetrachlorooxorhenate as rhenium precursor and complex forming ligands 1,2,3-trihydroxybenzene and 1,2-dihydroxybenzene. Borate buffers in the range of pH 6.0–11.5 were tested as background electrolytes, all of them provide complete resolution of all components of a reaction mixtures within 10 min. Effective electrophoretic mobilities of separated components were calculated and relevant graphs were designed. Optimal conditions for HPLC-UV/MS separation of reaction mixture components have been successfully discovered for the Agilent Zorbax SAX column, fully resolved peaks were identified by a mass spectrometry with electrospray ionization.

1. Introduction

Present nuclear medicine frequently uses transition metal based radiopharmaceuticals injected to patient's body in a form of short half-life radionuclide ion coordinated with suitable organic ligand [1]. Oxorhenium(+V) complexes composed of β-emitting rhenium isotopes (^{186}Re and ^{188}Re) and complex forming ligands start to play an important role in radiopharmaceutical medicine. Because of their similarity with already in medicine used technetium analogues, many tasks concerning the preparation and clinical utilization of corresponding formulations have been already solved. Coordination compounds of ^{99m}Tc are well established in nuclear diagnostics, ^{186}Re and ^{188}Re analogues seem to be utilizable in the bone antitumor therapy [2, 3].

Metal complexes tested and utilized in medicine can be characterized by a wide spectrum of analytical methods, thin layer chromatography, high performance

liquid chromatography, and capillary zone electrophoresis are preferred in practice [4–6].

In our previous contribution, we pointed out on suitability of low resolution ESI-MS for a fast and reliable structural identification of selected rhenium complexes in a mixture [7]. The goal of this study is to investigate simple methods for separation and determination of individual components in reaction mixture (unreacted precursor, ligand and resulting rhenium complex) using CZE/UV in an aqueous borate background electrolyte and complementary HPLC-UV/MS method using an Agilent Zorbax SAX column.

2. Experimental

2.1 Reagents and chemicals

Tetrabutylammonium-tetrachlorooxorhenate [(*n*-Bu_4N)($ReOCl_4$)] and ammonium formiate were purchased from Sigma-Aldrich. 1,2,3-trihydroxybenzene (PG) and 1,2-dihydroxybenzene (Cat) were purchased from Alfa Aesar. Acetonitrile HPLC grade was purchased from Lach-Ner. Triethylamine was purchased from Sigma-Aldrich. Boric acid, sodium tetraborate and sodium hydroxide were purchased from Lachema.

2.2 Instrumentation

A capillary electrophoresis system 7100 (Agilent Technologies) equipped with an on-line diodearray detector (DAD) operated in the range 200–600 nm, total time of 10 min. Experimental parameters for CZE measurements in borate buffer were set to: applied voltage 25 kV; hydrodynamic injection 30 mBar, injection time 6 s, passing current 12–30 μA; cassette temperature 25 °C; analysis time set to 10 minutes. The separation was performed in fused silica capillaries (50 cm, 41.5 cm to detector, 50 μm I.D.) (Polymicro TechnologiesA). Before the use, capillaries were rinsed with 100 mM aqueous NaOH (60 min) and deionized water (60 min). At the beginning of the day, the capillary was rinsed with water (10 min), 100 mM aqueous NaOH (10 min) and water (5 min). Prior to each analysis, the capillary was flushed with backround electrolyte (1.5 min) and after the separation with water (1.5 min) and background electrolyte (1.5 min).

For a HPLC chromatography the HP 1100 device with a quaternary pump and a DAD detector was used (Hewlet Parckard). Electrospray mass spectrometry experiments were conducted on an ion trap instrument Esquire 3000 in a negative ion mode at a scan range m/z = 300–650 and under conditions: nebulizer gas pressure 124.1 kPa, flow rate 5 L min^{-1}, the desolvation temperature was 300 °C and capillary voltage 4000 V; controlled by the Esquire Control 5.3.11; data were processed via Data Analysis 3.3.56 (Bruker Daltonics). The sample solutions were delivered to nebulizer by a syringe pump (Cole Parmer) at a flow rate 8 μL min^{-1}.

An Agilent Zorbax SAX 150×4.6 mm (5 μm), an ionex polar bonded-phase column used for a anion exchange in common organic solvents and aqueous buffer solutions (pH = 2.0–7.0), was used as the column. Anion retention depends mostly on pH of mobile phase and its ionic strength, less on column temperature, that should not exceed 40 °C.

2.3 Synthesis of the bis(1,2,3-trihydroxybenezen)-dioxorhenium(VII) complex

The reaction of 1.42 μmol of [(*n*-Bu_4N)($ReOCl_4$)] with 3.81 μmol of PG in 3 mL of acetonitrile was performed at room temperature, and after 10 min 12 equivalents of triethylamine (in molar excess in deoxygenated acetonitrile) was added. Resulting brown solution containing major Re(+VII)-PG (bis(1,2,3-trihydroxybenzene)-dioxorhenium) after 60 minutes of reacting. Mass values: theoretical monoisotopic 466.9 *m/z*; obtained 466.6 *m/z*.

2.4 Synthesis of the bis(1,2-dihydroxybenzene)-dioxorhenium(VII)

Reaction compounds, i.e. 1.0 μmol of [(*n*-Bu_4N)($ReOCl_4$)] and 2.0 μmol of Cat, were dissolved in 3 mL of acetonitrile at room temperature. After complete dissolution, 12 equivalents of triethylamine were added. Resulting deep magneta solution containing major Re(+VII)-Cat (bis(1,2,3-trihydroxybenzene)-dioxorhenium) after 60 minutes of reacting. Mass values: theoretical monoisotopic 434.9 *m/z*; obtained 435.5 *m/z*.

3. Results and discussion

3.1 Capillary electrophoresis

The pH value of background electrolyte is a key optimization factor for improving resolution of weak electrolytes, affecting both the overall charges of the solutes and the electroosmotic flow. As it is evident from Fig. 1, the effective electrophoretic mobilities of both ligands is significantly affected by pH. An effective electrophoretic mobility of a complex and perrhenate as a degradation product remains unchanged within studied pH range. Due to the presence of third hydroxyl group, PG shows gradual increase of effective mobility with increasing pH of BGE and higher maximum value, compared to catechol. The presence of unbounded hydroxyl group also increases effective electrophoretic mobility of rhenium-PG complex, in comparison to the rhenium-catechol one.

CZE was else utilized as a fast method for checking purity of Re(+VII)-Cat complex. As it is evident from Fig. 2, CZE proved to be sufficiently selective and sensitive, making it possible to quantify complex formed in a reaction mixture (part A) and check its purity after HPLC refinement (part B).

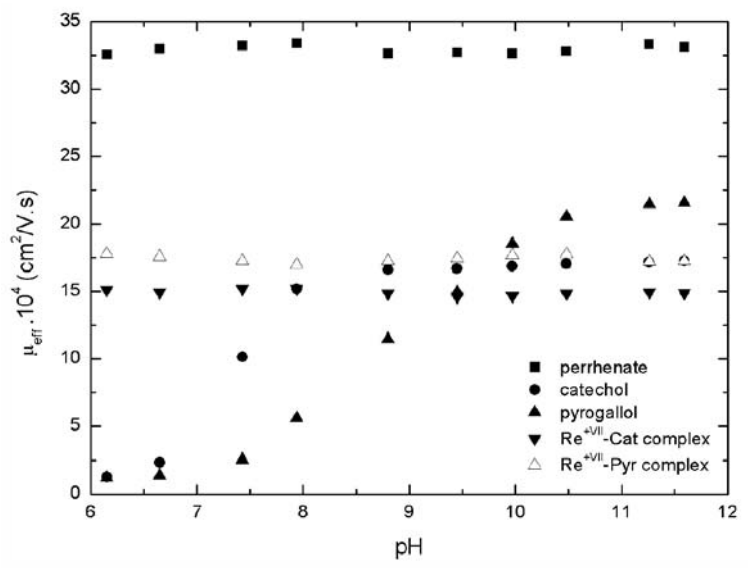

Fig. 1. Plot of the effective ion mobility of selected ions in reaction mixture depending on the pH value of borate buffer. CZE conditions: voltage 25 kV, current 25 μA, fused silica capillary (50 cm total length, 41.5 cm to detector, 50 μm I.D.), DAD detector 230 nm, hydrodynamic injections 30 mBar (30 s), cassette temperature 25 °C.

3.2 High performance liquid chromatography

3.2.1 Separation of Re(+VII)-PG complex

The effect of the mobile phase composition on the resolution of reaction mixture components was tested. It was found that the decrease of aqueous buffer content below 1% (*v/v*) leads to the decrease of mobile phase elution strength, resulting in significant increase of complex retention, accompanied with considerable peak deformation. When the buffer amount exceeds 8%, a co-elution of the complex and perrhenate appears. The optimal ratio of acetonitrile and buffer was determined as 93%:7%.

Due to the restricted stability of used stationary phase in alkaline media, it was possible to use only acidic or neutral mobile phases. With an increasing pH value, the elution strength of the mobile phase increases and the retention of the individual components decrease. Simultaneously, the chromatographic zones are being focused which improves the separation effectiveness. From point of view of optimal separation efficiency and resolution, isocratic elution with a mobile phase containing 7% of a buffer (pH = 7.6) and flow rate 1.0 mL min^{-1} was found to be optimal. Fig. 3 shows a chromatogram gained under such optimal conditions. Under optimal conditions (stated above), the following separation parameters were estimated for complex-ligand pair: separation factor α = 1.49; resolution R_s = 2.0, and the average number of theoretical plates n = 1225.

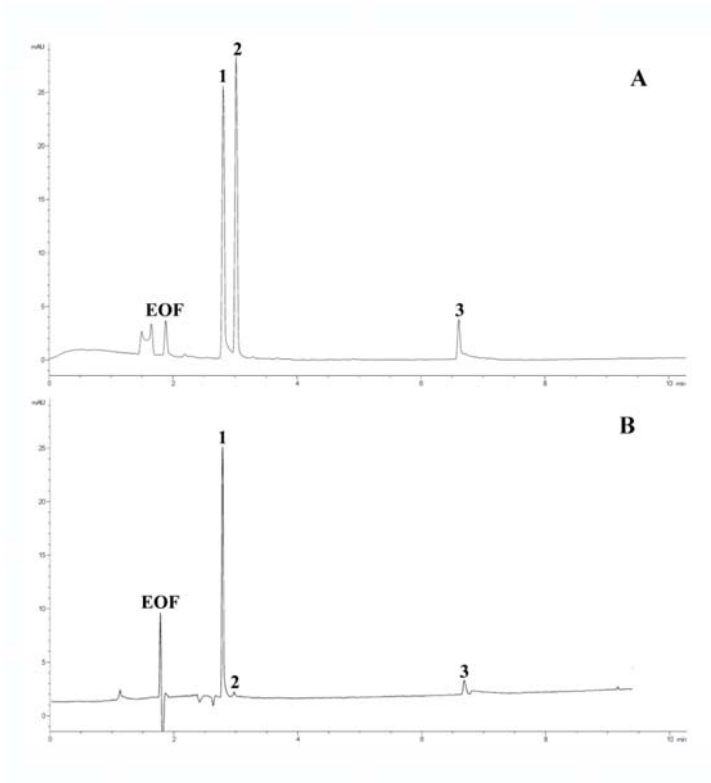

Fig. 2. Comparison of CZE records of reaction mixture with Re(+VII)-Cat complex (a) before, and (b) after semipreparative HPLC analysis. Peak identifications: (1) Re(+VIII)-Cat complex; (2) Cat; (3) perrhenate. CZE conditions: voltage 25 kV, current 20–22 μA, fused silica capillary (50 cm total length, 41.5 cm to detector, 50 μm I.D.), DAD detector 230 nm, hydrodynamic injections 30 mBar (30 s), cassette temperature 25 °C.

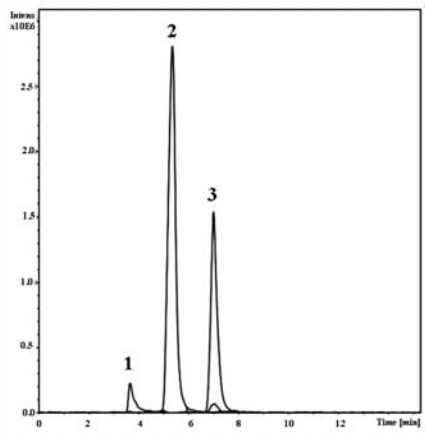

Fig. 3. HPLC-UV/MS chromatogram of selected ions: (1) PG (*m/z* 125); (2) perrhenate (*m/z* 251); (3) Re(+VII)-PG complex (*m/z* 467). Agilent Zorbax SAX column.

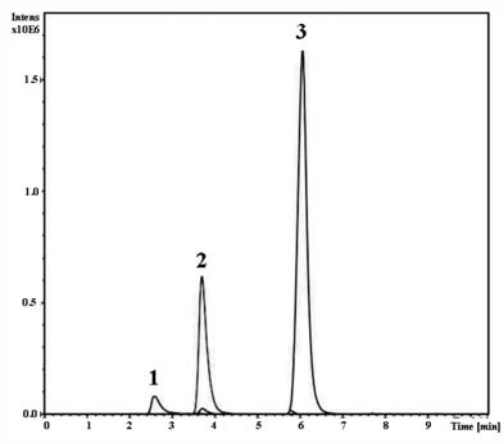

Fig. 4. HPLC-UV/MS chromatogram of selected ions: (1) Cat (*m/z* 109); (2) perrhenate (*m/z* 251); (3) Re(+VII)-PG complex (*m/z* 467). Agilent Zorbax SAX column.

3.2.2 Separation of Re(+VII)-Cat complex

The conditions of optimized PG complex HPLC separation are appropriate also for complex reaction with Cat. The retention factor for rhenium-catechol complex reaches roughly half of the values estimated for complex with PG, its elution occurs earlier and the corresponding peak is shown prior to the perrhenate peak. Analogically to Re(+VII)-PG complex, mobile phase with acidic buffer (pH value lower than 4), the elution times of perrhenate and complex are exceedingly prolonged, the peaks disappear from a chromatogram. The following conditions were found to be optimal: mobile phase composition ACN: FA ratio of 93%:7%; buffer pH = 7.6. The chromatogram obtained under optimum conditions is given in Fig. 4.

4. Conclusions

In summary, the methods developed in this study were successfully applied to the analysis of rhenium complexes with 1,2,3-trihydroxybenzene and 1,2-dihydroxybenzene. CZE in a borate buffer proved to be a useful tool for the fast monitoring of the complex forming reaction as well as for a effectiveness monitoring of a semi-preparative HPLC. HPLC with UV and MS detector using an Agilent Zorbax SAX column was used for analysis and micro-preparation of Re(VII) complexes.

Acknowledgments

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Analysis of perchlorate in baby food with high fat content

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Keywords
baby food
fat removal
liquid chromatography
perchlorate analysis
tandem mass spectrometry

Abstract
The aim of the present work was to compare three methods which can be applicable for the isolation of perchlorate anion from baby food with high fat content. Two methods (A, B) were based on QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) extraction but they differed in the extract purification. Third method (C) was based on QuPPe (Quick method for the analysis of highly Polar Pesticides) extraction which was modified by the addition of clean-up step. Analysis of perchlorate was carried out by the liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS). Within validation, all methods proved that they are suitable for the extraction of perchlorate. However, for further analyses of similar samples, method B was chosen because it provided better performance characteristics than method C and included simpler clean-up step than method A which led to the reduction of time and cost of the analysis.

1. Introduction

Perchlorates are salts of perchloric acid, most of them are soluble in water and circulate within the hydrologic cycle [1]. Perchlorates occurs naturally in deposits of nitrate and potash, and can be formed in the atmosphere and precipitate into soil and groundwater. Moreover, they are used in the metalworking industry, in paper upgrading and, as oxidants, for propellants in pyrotechnic materials [1, 2]. While industrial emissions are not expected to represent a main source of contamination in Europe, the use of natural fertilisers such as Chilean nitrate may lead to substantial concentrations of perchlorate in plants. Similarly, irrigation with contaminated groundwater can contribute to its accumulation in fruit and vegetable. Water disinfection with chlorine-based products, potentially degrading to perchlorate, could be another source of contamination [2]. Harmful effect of perchlorate on human health originates from its ability to inhibit the production of

thyroxine and triiodothyronine in the thyroid by blocking the uptake of iodide from the blood [1].

Perchlorate can act as a herbicide [1], but it is not approved within the European Union and there is no maximum residue level listed in Regulation (EC) 396/2005 [3]. In 2014, the European Food Safety Authority (EFSA) reported that the chronic dietary exposure to perchlorate is of a potential concern, in particular for the consumers in the younger age groups of the population with mild to moderate iodine deficiency [2]. In 2015, European Commission published a statement as regards the presence of perchlorate in food [4] and established reference levels for intra-Union trade. For foods for infants and young children (ready to eat), reference level is actually 0.02 mg/kg. Subsequently, Commission Recommendation (EC) No. 2015/682 [5] on the monitoring of the presence of perchlorate in food was adopted.

Ion chromatography coupled with conductivity detection or tandem mass spectrometry have been commonly used for the analysis of perchlorate [1, 6, 7]. Alternative method which uses reverse-phase HPLC on hydrophobic C18 column was firstly described by Li and George [6] for the analysis of water and subsequently by Tefera et al. [7] for juice and milk matrices and Hepperle et al. [1] for food samples of plant origin. To our knowledge, fewer investigations have been made in order to determine perchlorate in food samples with high fat content, such as particular kinds of baby food which contain meat ingredient together with vegetable and potatoes or rice. Thus, the aim of the present study was to compare three different methods which can be potentially suitable for the extraction of perchlorate from such kinds of samples.

2. Experimental

2.1 Reagents and chemicals

Sodium perchlorate (purity > 98 %) was purchased from Sigma Aldrich. Standard was dissolved in methanol to prepare stock solution with concentration of 0.1 mg/ml, whereas its purity and the sodium part were taken into account. The stock solution was diluted to 1 μg/ml (working standard) with methanol. Solutions were stored in the dark at 1 °C. Magnesium sulfate, sodium chloride, sodium citrate monobasic and sodium citrate dibasic sesquihydrate were of analytical grade, purchased from Sigma Aldrich. Bond Elut EMR-Lipid tubes (5982-1010) and Bond Elut Final Polish for EMR-Lipid tubes (5982-0101) as well as Bondesil-C18 sorbent were purchased from Agilent Technologies. Acetonitrile and methanol were of HPLC grade, purchased from Sigma Aldrich, formic acid was of analytical grade purchased from Merck. Samples of baby food were provided by their producer (he wishes to remain anonymous) and consisted from potatoes, carrot, chicken meat (10%), pea, and other minor components such as edible oil and potato starch.

2.2 Instrumentation

For the extraction of perchlorate, three different methods were used.

Method A applied QuEChERS (EN 15662) technique [8], which consisted from the extraction of 10 g of a sample by 10 ml of acetonitrile together with an addition of extraction salts, centrifugation and subsequent purification of upper layer (5 ml) via disper-sive solid phase extraction (dSPE). For dSPE, Bond Elut EMR-Lipid tubes and Bond Elut Final Polish for EMR-Lipid tubes were used according to the Agilent application note (5991-6098EN) [9].

In method B, also QuEChERS was used for the extraction, but for the purification of raw extract (1ml) only dSPE with Bondesil-C18 sorbent (150 mg) was applied.

Method C was based on QuPPe technique which consisted from the extraction of 10 g of sample by 10 ml of acidified methanol as it was described by Hepperle et al. [1] but after centrifugation, upper layer (1 ml) was further purified by dSPE with Bondesil-C18 sorbent (150 mg). For all extraction methods, 200 µl of purified extract was diluted by 800 µl of deionized water before analysis.

Perchlorate analysis was carried out using Agilent Technologies 1260 Infinity HPLC system coupled with an Agilent Technologies 6460 Triple Quad mass spectrometer. The analytical separation was performed on column Luna 3u C18(2) 100 A (50 mm×2 mm×3.2 µm) (Phenomenex) using an isocratic elution. Mobile phase consisted of 20:80 (*v/v*) mixture of methanol and 0.1 % formic acid in water, delivered at a flow rate 0.1 ml/min. The injection volume was 10 µl, the column temperature was set to 40 °C. The mass spectrometer operated with the electrospray ionization (ESI) source in the negative-ion mode. Multiple reaction monitoring (MRM) was performed for following transitions of perchlorate: 99 → 83 (quantifier) and 101 → 85 (qualifier). The optimized fragmentor voltage and collision energy were 140 and 30 V, respectively for both transitions.

3. Results and discussion

The main subject of perchlorate analysis is drinking water and soil [1]. Only few studies about its determination in food have been published and they were focused predominately on the vegetable and fruit [1, 6, 7]. With exception of Tefera et al. work [7] which dealed with the extraction of perchlorate from milk, no study regarding the analysis of perchlorate in matrices with high fat content have been carried out. QuEChERS is extraction method of choice for broad range of pesticides and there are a lot of its modifications in dependence on the character of sample which differed predominately in the clean-up step. Also for matrices with high fat content some QuEChERS protocols were designed but no one among them included perchlorate as one of the measured components. On the other hand, perchlorate was successfully extracted by QePPe method which was designed for the analysis of highly polar pesticides. Nevertheless, this method have not been

Table 1
Firuges of merits (limit of detection, limit of quantification, regression coefficient, repeatability, and reproducibility values of perchlorate anion) obtained for three studied extraction methods.

Characteristics	Method A	Method B	Method C
R^2	0.99811	0,99801	0.9916
LOD [mg/kg]	0.0013	0.0015	0.0019
LOQ [mg/kg]	0.0024	0.0027	0.0034
RSD_r [%]	3.7	4.0	7.5
RSD_R [%]	6.1	6.9	8.6

used for the samples with high fat content. QuPPe does not include any purification of raw extract, only its filtration and dilution and this can lead to the complications during the analysis.

In this study, we modified QePPe method (C) via addition of purification step on the basis of dSPE, with C18 applied as sorbent and compared it with two QeChERS methods. One of them (A) used commercially available kit for the purification of raw extract consisting from two mixtures of sorbents (with unknown composition) weighted into the extraction tubes. Second QeChERS method (B) used only C18 sorbent for dSPE. For all three methods, parameters such as linearity, LOD and LOQ values, repeatability and reproducibility were calculated.

The standard addition method was used for the quantification of perchlorate to control the influence of the sample matrix. To establish the calibration, working standard of perchlorate was gradually added directly into the samples of baby food at content ranged up 0.001 to 0.1 mg/kg. Spiked samples were extracted by all methods and analysed. Linearity of detector reponse was checked through calibration curves which were obtained by the linear regression of the peak area versus concentration of perchlorate in injected extracts. The calculated coefficients of determination were above 0.99 for all three methods (Table 1). Limit of detection and limit of quantification were calculated using the program Mass Hunter Workstation software (Version B. 04.00), obtained values ranged between 0.0013 and 0.0019 mg/kg for LOD and between 0.0024 and 0.0034 mg/kg for LOQ. Precision of the methods was assessed by the calculating of the relative standard deviations in repeatability and reproducibility conditions. For evaluation of repeatability, samples of baby food were analysed after spiking at content 0.01 mg/kg in five replicates. For reproducibility, analyses of spiked baby food at concentration level 0.01 mg/kg were carried out by the different analysts. As follows from Table 1, these values were below 10% for all three methods, whereas RSDr values ranged between 3.7 and 7.5%, and RSDR values ranged between 6.1 and 8.6%. Finally, all methods were tested via the analyses of three samples provided by their producer in which the content of perchlorate was determined by the independent laboratory. Our results were in good agreement with the provided data (Table 2).

Table 2
Results of analyses (average values [mg/kg] calculated on the basis of experimental data) of perchlorate in samples of baby food and their comparison with the data from independent laboratory provided by the producer of the samples.

Sample	Independent laboratory	Method A	Method B	Method C
1	0.0040±0.0008	0.0030±0.0008	0.0029±0.0007	≤ LOQ
2	0.033±0.008	0.042±0.005	0.045±0.006	0.023±0.006
3	0.028±0.007	0.021±0.007	0.032±0.006	0.018±0.009

When compared results obtained in the Table 1, it is obvious that although all methods passed the validation, the method C provided the worst performance characteristics, close to the edge of acceptance. This is probably due to the larger amount of matrix ingredients coextracted by methanol in comparison with other methods in which acetonitrile was used. Coextracted matrix components resulted in strong interferences which were observed in the form of uneven peaks, predominately during the analyses of samples spiked at lower concentrations. In this case, the purification of extracts by dSPE with C18 sorbent was insufficient and use of more efficient clean-up such as some type of SPE cartridges or different sorbents would be appropriate. Method A provided the best performance characteristics and confirmed efficiency of commercially available mixtures of sorbents for clean-up of perchlorate extracts. However, this method needs more sample handling and longer time (it involves two centrifugations) and also the price of used sorbents is not negligible. Method B provided analytical characteristics comparable with method A and because of the simpler and shorter clean-up step along with lower amount and price of used sorbent, it was chosen for the further analyses of similar samples. Fig. 1 represents HPLC-MS/MS chromatograms of perchlorate transitions in the extract of baby food spiked at concentration 0.01 mg/kg obtained by this method.

4. Conclusions

On the basis of experimental data, it is possible to postulate that all evaluated extraction methods are suitable for the isolation of perchlorate anion from baby food samples with high fat content. However, analyses of extracts obtained by method C (based on QuPPe extraction) provided uneven peaks of perchlorate at lower concentrations, probably due to the strong matrix interferences which resulted in the worst performance characteristics among all tested methods. It indicates that use of more effective clean-up would be appropriate in this case. Results of validation achieved for methods A and B (based on QueChERS extraction) were comparable but method B applied simpler clean-up step which reduced time of analysis and handling the sample, minimize consumption of used chemicals and significantly lowered cost of the analysis. Because of that, method B was chosen for further analyses of similar samples.

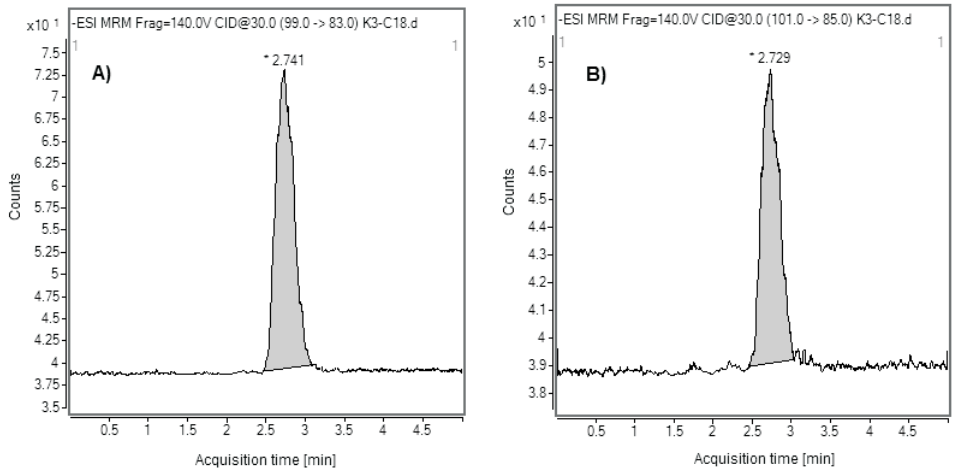

Fig. 1. HPLC-MS/MS chromatograms of perchlorate MRM transitions (A: 99 → 83, and B: 101 → 85) in the extract of baby food spiked at concentration 0.01 mg/kg obtained by the method B.

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Biosensors with protective membranes for an electrochemical study of the interactions of DNA intercalators in biological matrix

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Keywords
complex biological matrix
DNA based biosensor
DNA intercalators
external protective membrane

Abstract
Electrochemical DNA based biosensors with textured surface have been prepared for the investigation of DNA interactions with specific chemicals in biological samples of complex matrix such as urine. Results obtained at the search and optimization of polymer protective films on the DNA recognition layer will be presented. Polyvinyl alcohol membrane has been found as effective barrier able to stabilize the biosensor response in the given matrix. An interaction of acridine yellow and doxorubicin as representatives of the DNA intercalators has been studied by using cyclic voltammetry with the DNA redox indicator and square wave voltammetry of the guanine and adenine moieties anodic responses.

1. Introduction

The electrochemical DNA based biosensor is an analytical device that represents the connection between the nucleic acid as a biorecognition element and the electrode made of different materials as a physicochemical transducer [1]. External protective polymer membranes have been reported to prevent the biosensor surface against the influence of interfering substances in complex matrices [2].

Intercalators are the significant class of aromatic compounds and some of them are used in the treatment of cancer. Acridine yellow is a yellow dye which damages DNA and is used as a mutagen in the microbiology. Studies show that acridine yellow is the intercalator capable of binding to the DNA double helix and change its structure [3]. Sakomoto [4] also shows possibility of the electrochemical determination of DNA damage in the presence of derivate of acridine. Doxorubicin also called daunomycin or adriamycin is an anthracycline antibiotic. It is known that doxorubicin interacts with DNA and inhibits the function of the enzyme topoisomerase II [5]. There are also studies of the electrochemical determination of doxorubicin and its effect on the DNA structure using biosensors [6].

The goal of the work was to compare the possibility of the detection and monitoring of the double-stranded (dsDNA) interaction with intercalating substances in phosphate buffer and in complex biological matrix of urine.

2. Experimental

2.1 Reagents and chemicals

Salmon sperm dsDNA was obtained from Sigma-Aldrich. Its stock solution (0.1 mg ml^{-1}) was prepared in a phosphate buffer of pH = 7.4 and stored at 4 °C. Polyvinylalcohol (PVA) was obtained from Sigma-Aldrich, and dissolved it nanopure water to the concentration 0.1 mg ml^{-1}. Stock solutions of acridine yellow and doxorubicin were of concentration 2×10^{-5} mol dm^{-3}.

2.2 Instrumentation

All measurements were performed using the potentiostat Autolab and were carried out in the three-electrode system using a glassy carbon working electrode (GCE), a silver|silver chloride reference electrode (Ag/AgCl/sat KCl) and a platinum counter electrode.

Cyclic voltammetry (CV): in 1 mM $[Fe(CN)_6]^{3-/4-}$ in 0.1 mol dm^{-3} phosphate buffer, potential range from –0.4 to 0.8 V, scan rate 100 mV s^{-1}, potential step 2 mV.

Square wave voltammetry (SWV): in 0.1 mol dm^{-3} phosphate buffer pH = 7.4, pulse amplitude 40 mV, frequency 100 Hz, scan rate 1.5 V s^{-1}, potential step 15 mV.

Electrochemical impedance spectroscopy: in 1 mM $[Fe(CN)_6]^{3-/4-}$ in 0.1 mol dm^{-3} phosphate buffer, polarization potential 0.1 V, frequency range from 0.1 Hz to 5000 Hz (51 frequency steps), amplitude 10 mV.

2.3 Preparation of the biosensors

The GCE surface was mechanically cleaned by using of "alumina slurry" and pretreated by applying a constant potential of 1.6 V. Chemical modification of the electrode was carried out by covering the surface of the GCE by a layer of dsDNA deposited on the electrode surface under the conditions of the adsorption potential to obtain the DNA modified electrode DNA/GCE. Then, a drop of PVA stock solution was deposited and let to evaporate to dryness to obtain the PVA/DNA/GCE biosensor. Prior to measurement, all working electrodes were immersed in PB for 2 min under stirring to achieve equilibrium.

3. Results and discussion

CV measurements indicated that the interferences in the urine were adsorbed on the surface of both the bare GCE and the DNA/GCE electrodes and reduced the redox response of the $[Fe(CN)_6]^{3-/4-}$ indicator. Several polymers have been tested

to protect the electrode surface. From the evaluation of the time and dimensional stability of the CV curves of the redox indicator it was concluded that the PVA membrane is suitable for the protection of biosensor from high molecular weight substances in the urine. This was also confirmed by electrochemical impedance spectroscopic (EIS) measurements. From the SWV measurements it was found that the membrane does not change significantly the signals of adenine and guanine base moieties.

The DNA biosensor with the PVA protective membrane (PVA/DNA/GCE) was used to test an effect of the known intercalating substances acridine yellow and doxorubicin added to the urine matrix on the structure of immobilized DNA. The CV measurements confirmed the intercalation of the substances under study and the SWV measurements showed a significant change in the structure of DNA after an incubation of the biosensor in solutions of intercalating substances. The intercalation has been detectable in buffer alone and in the biological matrix as well.

4. Conclusions

The goal of the work was to construct the electrochemical DNA based biosensor with a polymer membrane for the analytical evaluation of substances interacting with DNA in a complex biological fluid. It was found that the electrochemical analysis using the biosensor with protective membrane is suitable for the investigation of interaction of the intercalators such as acridine yellow and doxorubicin with dsDNA in urine and confirmation of DNA structural changes under such conditions.

Acknowledgments

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Development of chromatographic and mass spectrometry conditions for untargeted lipidomic analysis of human breast milk

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Keywords
human breast milk
lipidomic study
liquid chromatography
time-of-flight mass spectrometry

Abstract
Human breast milk is a complex biofluid containing components which are essential for the proper growth of the newborn. The molecular composition of lipids in human breast milk is not well known as the protein content. The aim of this study was to develop methodology for untargeted lipidomic analysis of human breast milk. Analysis of human breast milk lipids was carried out with use high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (LC-Q-TOF-MS). Methods for sample preparation, separation and identification of total lipids present in human breast milk during one analytical cycle are presented.

1. Introduction

Human breast milk is a complex biofluid which composition depends on many factors. Human breast milk not only contains nutrients (lipids, proteins, carbohydrates, and minerals) which are necessary for right growth of the infant but also bioactive substances (hormones, antibodies, antimicrobial substances) which protect the infant against infection, inflammation, and contribute to maturation of the immune system [1, 2]. Because of its content, human breast milk is the only food able to satisfy all the nutritional needs of the newborn child, by providing all the nutrients required for somathic growth and also important functional factors [3, 4]. Until now, several studies involving human breast milk investigations were performed, including determination of substances that are essential for right growth of an infant (such as antibodies, hormones, nutrients) and undesirable chemicals (such as parabens, phenols, flame retardants, chlorinated organic chemicals) [5–10]. In the case of nutrients investigations focussed mainly on the determination of proteins. The molecular composition of lipids in human breast milk is not so well known as the protein content. Lipids contain in the human breast milk are essential components for the infant (e.g., the largest source of

energy, influence on proper neurological development [3, 11]), therefore in-depth assessment of lipid's composition of human breast milk is reasonable. Development of comprehensive untargeted lipidomic methodology for human breast milk analysis also will provide the possibility to indicate variation in the composition of milk lipids (i.e., related to the different stage of lactation) so it will be an important contribution to the knowledge about chemical components contained in human breast milk.

The aim of this study was to develop a methodology for comprehensive untargeted lipidomic analysis and to demonstrate the usefulness of high performance liquid chromatography coupled with high resolution time-of-flight mass spectrometry for untargeted lipid profiling in human breast milk.

2. Experimental

2.1 Chemicals and reagents

LC-MS grade methanol, HPLC grade chloroform, and HPLC grade hexane were purchased from Merck, 2-propanol and ammonium formate (99.9% purity) were purchased from Sigma-Aldrich. Deionized water was purified by an HLP5 system (Hydrolab, Poland).

2.2 Sample preparation, extraction procedure

Milk samples were collected from the volunteers and stored in the special storage bags (dedicated for breast milk) in –80°C until analysis. Sample preparation was based on the extraction of lipids contained in human breast milk. The modified extraction procedure presented in [12] was applied for the crude lipid extract preparation. In the first step 450 µl of human breast milk was transferred to a borosilicate glass tube with a Teflon screw cap (Sigma Aldrich). Secondly, 1.9 ml mixture of chloroform and methanol (1:2, *v/v*) was introduced and followed by vortexing for 5 min. Next, 625µl of chloroform was added, followed by vortexing for 10 s and then 625 µl of deionized water was appended and followed by 60 s of vortexing. Subsequently, the sample was centrifuged at 4.4 rpm for 10 minutes. Finally, the organic phase containing lipids was gently aspirated with a glass Pasteur pipette and moved to a new glass tube. Afterwards, lipid extract was analyzed using LC-Q-TOF-MS.

2.3 Instrumentation

The RP-LC -Q-TOF-MS analysis was performed using Agilent 1290 LC system equipped with a binary pomp, an online degasser, an autosampler, and thermostated column compartment coupled with a 6540 Q-TOF-MS with a Dual electrospray ionization source (Agilent Technologies). An Agilent

ZORBAX SB C 18, 50×2.1mm, 1.8 µm column with 0.2 µm in-line filter was used for lipid separation. A mobile phase consisted of: mixture of 5mM ammonium formate in water and methanol (1:9, *v/v*) (component A) and mixture of 5mM ammonium formate in water, hexane, 2-propanol (1:20:79, *v/v/v*) (component B). The following gradient elution program was applied: 0% B during first five minutes, from 0% to 40% from 5 to 28 minutes, 40–100% B during minutes 28–32, followed by 100% B and was maintained for 3 minutes. Subsequently, after 1 minute, the composition of eluent was returned to 0% B and with this mobile phase composition 10 min of equilibration was performed before the next injection. The column temperature during the chromatographic separation process was maintained at 90 °C. The mobile phase flow rate was 0.6 ml/min and the injection volume was 0.5 µl. Throughout the analysis, the samples were kept in an autosampler at 4 °C.

The electrospray ionization source was operated in positive ion mode. The condition parameters were set to the following: the fragmentor voltage at 120 V, nebulizer gas at 35 psi, capillary voltage at 3500 V, drying gas flow rate at 10 l/min and temperature at 300 °C. Data were aquired in centroid and profile mode using High Resolution mode (4 GHz). The mass range was set at 200–1700 *m/z* in MS mode. The TOF was calibrated on a daily basis, before the beginning of analysis.

To monitor and exclude compounds, which do not originate from human breast milk but fairly from the chemicals used for extraction or LC-MS analysis, blank samples were used. Preparation of blank samples was exactly the same as in the case of the studied samples (extraction procedure with deionized water instead of human breast milk).

The proposed workflow for untargeted lipidomic analysis of human breast milk, including all analytical steps is shown in Fig. 1.

2.4 Data analysis

Raw data obtained from RP-LC-Q-TOF-MS system were processed with the MassHunter Workstation Software Qualitative Analysis, B.03.01 version (Agilent Technologies). Parameters used for molecular feature extraction were as follows: extraction algorithm, small molecule; input data range, restricted retention time 1–28 min, restricted *m/z* = 450–1700; peak filters; peak with hight ≥ 2000, ion species, +H, peak spacing tolerance 0,025 *m/z* plus 7.0 ppm; isotope model, common organic molecules and charge state, 2. The result of molecular feature extraction was a list of all molecular entities, which present the full TOF mass spectral data for each sample.

Identification of lipids compounds was performed by comparing the mass accuracy of obtained MF against online database *LipidMAPS* (www.lipidmaps.org). Mass error was set to 5 ppm. To confirm the identity of the lipid compounds, MS/MS experiments were done. The obtained MS/MS spectra were compared with fragmentation pattern of lipid compounds.

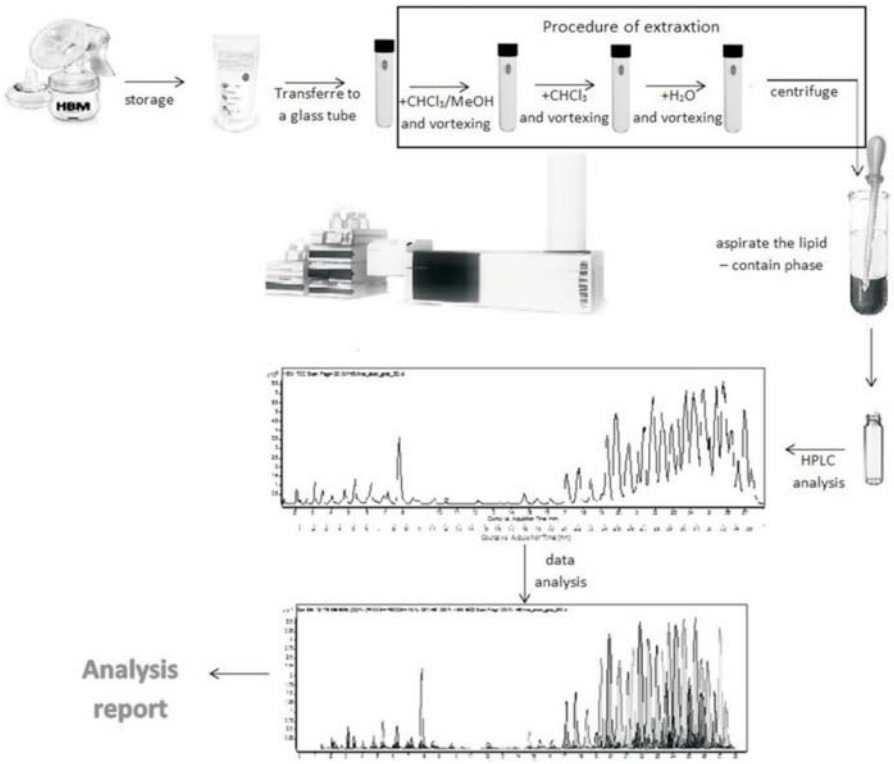

Fig. 1. A simplified workflow for the lipidomic study of human breast milk.

3. Results and discussion

In the present study an untargeted lipidomic analysis using LC-MS was performed. Development of comprehensive method for lipidomic studies is challenging because of the large number of lipid species and their structural diversity (polar and nonpolar compounds) and also due to co-eluting isobaric and isomeric species. The developed method should provide high discrimination power, cover the substantial portion of the human breast milk lipidome and be characterized by high precision and accuracy to allow indication of even small lipidomics differences between analysed samples.

The chromatographic and MS determination condition were optimized to achieved both high resolution and abundance of human breast milk lipids. It should be noted that lipid components strongly vary in lipophilicity (i.e., high hydrophobic triacylglycerols, polar glycerophospholipids), therefore, their separation is not so easy. Thereupon, several solvents and compositions of mobile phase were investigated during optimization process. The best chromatographic resolution and efficient strength of mobile phase were provided by the mixture of 5 mM ammonium formate in water and methanol (1:9, *v/v*) (component A) and mixture

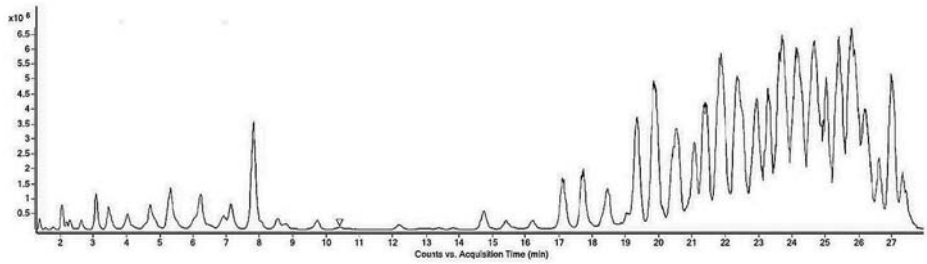

Fig. 2. Total Compounds Chromatogram obtained as a result of data processing with the use of molecular feature extraction algorithm.

of 5mM ammonium formate in water:hexane:2-propanol (1:20:79, *v/v/v*) (component B). Parameters such as flow rate, gradient step and column temperature were optimized. Initial chromatographic conditions were taken from paper published in [12]. Originally, time of analysis and column temperature were 71 minutes and 45 °C, respectively. Unfortunately, this chromatographic conditions did not guaranteed the elution of the most hydrophobic compounds (triacylglycerols), but the increase of temperature from 45 °C to 90 °C enabled elution of all lipids without any carryover effects. To reduce analysis time, flow rate was change from 0.3 ml/min to 0.6 ml/min. Increase of flow rate ensured reduction of analysis time to 36 min. Simultaneously with the chromatographic conditions, MS working parameters were optimized to obtain high MS signal intensity and also to detect the largest number of molecular features.

The total ion chromatogram obtained during the HPLC-MS analysis of human breast milk lipid extract was processed by the algorithm of molecular feature extraction that allows to exclude a background noise from chromatograms and to obtain a list of all molecular entities, including the full mass spectral data for each compound. The resulting molecular feature extraction chromatogram (total compounds chromatogram) is shown in Fig. 2.

Application of tentative identification of lipids (achieved by comparing the mass accuracy of MF against online database *LipidMAPS*) and MS/MS experiments (confirms the identity of the lipid compounds), enabled identification of lipid classes such as diacylglycerols, triacylglycerols, glycerophospholipids, and sphingomyelines. Exemplary extracted compounds chromatogram of human breast milk sample is shown in Fig. 3.

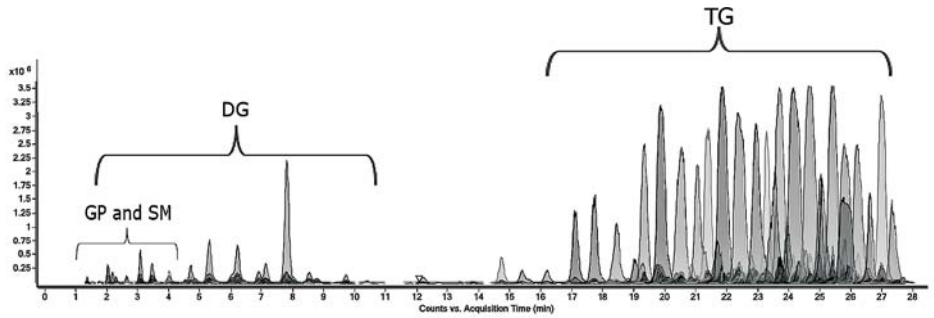

Fig. 3. Extracted compound chromatograms of all compounds obtained by LC-Q-TOF-MS analysis of human breast milk lipid extracts with indication of elution order of main lipid classess identified in human breast milk: (GP) glycerophospholipids, (DG) diacylglycerols, (TG) triacylglycerols, (SM) spingomyelines.

4. Conclusions

The methodology for human breast milk untargeted lipidomics is a powerful tool that provides insight into the lipid composition of human breast milk. The application of a LC-Q-TOF-MS allowed to separate and identify major human breast milk lipid classes: diacylglycerols, triacylglycerols, glycerophospholipids, and sphingomyelines.

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Determination of biogenic amines in beers by LC-MS/MS

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Keywords
biogenic amines
derivatization
high performance liquid chromatography
tandem mass spectrometry
tosyl chloride

Abstract
Analysis of biogenic amines in beers was carried out by HPLC-MS/MS after their derivatization with *p*-toluenesulfonyl chloride (tosyl chloride). The developed method has been applied for analysis of eighteen biogenic amines in twelve samples of different kind of beers (Lager, Strong Lager, Schwarzbier, Weizenbier, Pils, nonalcoholic Pils, Stout). The method is sensitive and the results precise for majority of the analytes.

1. Introduction

Biogenic amines are a group of compounds that naturally occur in food and beverages as a result of free amino acids bacterial decarboxylation [1, 4]. Biogenic amines could be indicators of freshness and food quality, because their content is associated with food fermentation and degradation [3]. What's more, in food and beverages, concentration of biogenic amines may reach levels at which they become toxic to humans, so their determination is very important. The most important biogenic amines, occurring in food (e.g., cheese, meat) and beverages (e.g., wine, beers) are histamine, cadaverine, agmatine, tryptamine, tyramine, spermine, spermidine, and β-phenylethylamine [2]. Their structures are depicted in Fig. 1.

Interest in biogenic amines is connected with their influence on the human's nervous and vascular system [5]. Biogenic amines may cause hypertensive crisis (tyramine), allergic and migraine reactions (histamine), rash, nausea, and anaphylactic shock; mainly if biogenic amines rich food and monoamine oxidase inhibitors are consumed at the same time [4, 5]. What's more, secondary amines, such as: agmatine, spermine, and spermidine may form nitrosamines by reaction with nitrites [1, 5].

Toxic impact of biogenic amines usually is not connected with their high content in beer, but with a very high consumption of beer in a very short time. Therefore it is extremely important to control the concentration of biogenic amines in beers [1]. One of the most important reason for determination of

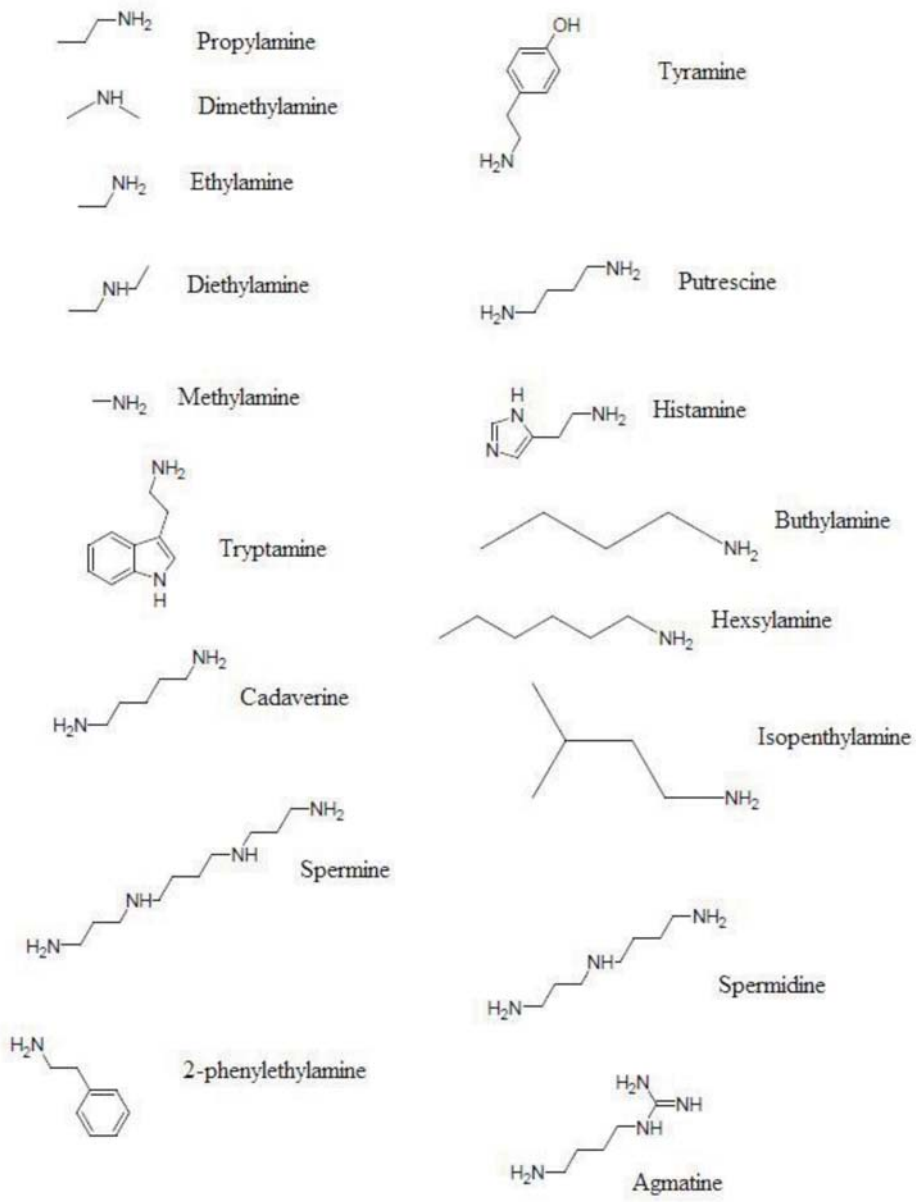

Fig. 1. Chemical structures of studied biogenic amines .

biogenic amines in alcoholic drinks is the fact that ethanol can suppress monoamine oxidase, thus inhibiting decomposition of biogenic amines in organism [8]. Moreover, biogenic amines fingerprints may be used to track the origin and authenticity of beers.

Determination of biogenic amines often causes difficulties, because of their polar nature and low levels of concentrations in food and beverages [3]. In order to

determine biogenic amines numerous kinds of analytical techniques like gas chromatography, thin layer chromatography, capillary electrophoresis, and high performance liquid chromatography had been used. HPLC seems to be one of the most frequently used techniques because of high sensitivity, resolution, relatively simple sample preparation and great versatility [3, 6, 7]. In addition, multiple reaction monitoring mode HPLC-MS/MS provides highly sensitive and selective detection.

In the case of HPLC, determination of biogenic amines in their native state is problematic due to severe peak tailing and low sensitivity. Therefore many derivatization agents have been used to improve their chromatographic behavior. Commonly used derivatization agents are: benzoyl chloride, dansyl chloride, *N*-(2-acridonyl)-maleimide, dansyl chloride, *o*-phthalaldehyde, and tosyl chloride. It is worth noting, that tosyl chloride is often used to derivatize polyamines but in the case of monoamines its use is not widespread [3, 6, 7]. The aim of study was to develop an LC-MS/MS method for determination of biogenic amines in beer after their derivatization with tosyl chloride.

2. Experimental

2.1 Chemicals and reagents

Eighteen biogenic amines; propylamine, dimethylamine, ethylamine, diethylamine, methylamine, tryptamine, cadaverine, spermine, 2-phenylethylamine, tyramine, putrescine, histamine, butylamine, hexsylamine, isopenthylamine, isobuthylamine, spermidine, agmatine, acetonitrile (LC-MS grade) and tosyl chloride (≥ 99%) were purchased from Sigma-Aldrich. Formic acid was purchased from Merck. Boric acid and sodium hydroxide were purchased from POCH (Gliwice, Poland). Ultrapure water was prepared using HLP5 system from Hydrolab (Wiślina, Poland). Borate buffer was prepared by titrating 0.5 M boric acid solution with sodium hydroxide to the required pH value.

2.2 Instrumentation

The HPLC-MS/MS analyses were performed using an Agilent 1200 LC system equipped with binary pump, a thermostat, an autosampler and online degasser coupled with an AB Sciex 4000 QTRAP mass spectrometer. The thermostat module was equipped with a switching valve. Gemini C-18 (150×4.6 mm, 3 μm, Phenomenex) column was used to separate analytes. Acetonitrile (component B) and water (component A) both with addition of formic acid (0.1% v/v) were used as the mobile phase. The gradient elution was as follows; 20% of B for 2 minutes, then linear increase to 65% B during 15 minutes, 65% B maintained for 3 minutes, increase from 65% B to 95% B during 3 minutes followed by 95% B maintained for 3 minutes. The last step was conditioning of the column for 3 minutes with 20% B.

During the first two minutes of a run the eluate was directed to waste by means of switching valve. Flow rate of mobile phase was 1 mL/min. The column temperature was maintained at 40 °C and the injection volume was 10 µl. The ESI source was operated in positive ion mode, source temperature was set at 500°C, press of nebulizer gas at 45 psi, heater gas at 30 psi, curtain gas at 20 psi, and ionspray voltage at 5000 V. Source and MS parameters are shown in Table 1. Analyst Software version 1.5.2 (AB Sciex, CA, USA) was used to acquire chromatograms and control instrumentation.

Table 1
Parameters of multiple reaction bonitoring for studied biogenic amines determination.

Compound	MRM	Declustering potential (V)	Collision energy (V)
Methylamine	186.101 → 155	71	17
	186.101 → 91	71	29
Dimethylamine	200.121→ 155	76	25
	200.121→ 91	76	37
Ethylamine	200.121→ 155	76	25
	200.121→ 91	76	37
Propylamine	214.095→155.10	71	23
	214.095→91	71	37
Buthylamine	228.144→155	66	23
	228.144→91	61	37
Isobuthylamine	228.144→155	66	23
	228.144→91	61	37
Diethylamine	228.144→155	66	23
	228.144→91	61	37
2-phenylethylamine	276.127→105	66	23
	276.127→77	66	69
Isopenthylamine	242.157→155	66	27
	242.157→91	66	43
Tryptamine	315.060→144.10	51	17
	315.060→117	51	77
Cadaverine	411.10→240.20	96	23
	411.10→184.10	96	29
Putrescine	397.11→226.30	96	23
	397.11→155.10	96	35
Spermidine	608.284→383.20	91	31
	608.284→226.30	106	37
Spermine	819.336→212.100	126	49
	819.336→281.100	126	51
Tyramine	446.172→275.100	101	21
	446.172→155.000	101	35
Histamine	420.095→109.000	81	35
	420.095→91.000	81	67
Agmatine	439.218→155.200	121	37
	439.218→91.000	121	71

2.3 Samples and sample preparation

Twelve bottles of different beers (Lager, Strong Lager, Schwarzbier, Weizenbier, Pils, nonalcoholic Pils, Stout) were purchased at local supermarkets. All beers were analysed within one day from purchase. All beer samples were degassed in an ultrasonic bath for 10 minutes and diluted (1:4, *v/v*) with ultrapure water.

In order to perform derivatization, 500 µl of diluted beer sample was transferred to a 12 ml glass test tube and mixed with 250 µl of borate buffer (0.5 M, pH = 11.0) and 500 µl of tosyl chloride solution (10 mg/mL in acetonitrile). After mixing, samples were incubated for 120 min at 50 °C in a water bath. Finally, the samples were filtered through a 0.2 µm nylon filter (Agilent Technologies) and injected into the chromatographic system.

2.4 Calibration curves

Stock solutions (1mg/mL) of single biogenic amines were prepared in 0.1 M HCl. Appropriate aliquots of these solutions were mixed together in a 25 mL volumetric flask and made up to the mark with acetonitril:0.1 M HCl (3:7, *v/v*) mixture in order to produce the standard mix. The concentrations of particular biogenic amines were adjusted based on preliminary analyses of samples being studied. Six-point (each point in triplicate) calibration curves were prepared by diluting variable aliquots of the standard mix with acetonitril:0.1 M HCl (3:7, *v/v*) mixture.

Table 2
Calibration ranges, correlation coefficient, *LOD*, and *LOQ* for studied biogenic amines determination.

Compound	Calibration range [µg/L]	R^2	*LOD* [µg/L]	*LOQ* [µg/L]
Dimethylamine	0.9–16.8	0.9996	0.31	1
Methylamine	18.4–331	0.9988	11	315
Ethylamine	22–198	0.9956	15	46
Diethylamine	1.23–11.1	0.9915	1.2	3.6
Propylamine	21.7–390	0.9988	12	37
Buthylamine	12.6–176	0.9973	9.4	28
Isobuthylamine	0.26–3.7	0.9967	0.26	0.81
2-phenylethylamine	5.14–92.5	0.9991	2.5	7.6
Isopenthylamine	1.28–23	0.9989	0.72	2.1
Putrescine	40.7–733	0..9981	29	88
Spermidine	7.5–136	0.9988	5.5	17
Spermine	32.5–29.2	0.9984	14	41
Tyramine	18.7–33.7	0.9999	9.7	29
Tryptamine	10.1–182	0.9985	3.6	11
Histamine	18.1–163	0.9988	13	40
Hexsylamine	20.5–369	0.9999	11	32
Agmatine	203–3650	0.9991	99	298
Cadaverine	16.2–292	0.9994	6.7	20

100 μL of a particular calibration solution was mixed with 400 μL of water and derivatized applying the same procedure which was used for beer samples. Calibration ranges for all compounds under the study, together with corresponding coefficients of determination and LOD and LOQ values calculated from calibration graphs are given in Table 2.

3. Results and discussion

3.1. Optimization of the separation and derivatization conditions

In order to separate derivatives of eighteen biogenic amines, the gradient elution program was developed. Best results, in terms of peak shape and sensitivity were obtained with mobile phase consisting of acetonitrile and water acidified with formic acid. Column temperature set to 40 °C was found beneficial due to lower system's backpressure.

Derivatization conditions were optimized mainly in terms of repeatability. The parameters being optimized were: derivatization time and temperature, volume and concentration of derivatization agent. It was noted that among compounds under the study tryptamine and diethylamine were the most problematic ones. For these compounds derivatization time as long as 120 minutes seems necessary to obtain repeatable results. It was also found that quenching derivatization reaction with 1 M HCl as suggested by Dziarkowska K. et al. [9] leads to unstability of biogenic amines derivatives caused most probably by their hydrolytic degradation at pH around 2.0.

3.2. Validation of method

Six-point calibration curves were prepared in the ranges shown in Table 2. In all cases, linearity is satisfactory with correlation coefficients R^2 >0.99. Limits of detection (*LOD*) were calculated as: *LOD*=3.3 × standard deviation of the intercept of the calibration curve / the slope of the calibration curve and the limits of quantification were calculated as three times LOD.

Repeatability of the method was estimated from triplicate analyses of beer samples (Table 3). As shown in Table 3, value of standard deviation for most of biogenic amines is relatively low, therefore it can be concluded that the method is repeatable and certainly can be used to determination of biogenic amines in beers. Only in the case of diethylamine, standard deviation was above criteria of acceptance, therefore must carry out a further study on a method for determination of biogenic amines in beers.

Table 3
Content of studied biogenic amines in analzsed beers given in [μg/l] ± standard deviation (*n*=3).

Compound	Strong lager	Weizen 1	Non-alcoholic 1	Schwarzbier 1	Pils	Non-alcoholic 2
Dimethylamine	2.58±0.12	1.157±0.023	0.817±0.066	3.190±0.010	3.660±0.014	6.83±0.32
Methylamine	21.8±1.4	7.91±0.35	13.47±0.21	27.13±0.29	18.8±3.8	13.1±0.9
Ethylamine	1067±38	578±23	674±35	1670±40	730±274	293±43
Diethylamine	N/A	N/A	N/A	0.69±0.36	N/A	0.32±0.35
Propylamine	N/A	N/A	N/A	N/A	N/A	N/A
Butylamine	111±12	73.37±7.05	54±15	166±14	84.2±7.1	62.4±9.7
Isobuthylamine	2.79±0.53	N/A	3.08±0.71	4.89±0.98	12.80±0.57	N/A
Phenylethylamine	3.15±0.19	1.07±0.28	2.21±0.47	12.1±0.7	13.20±0.14	0.37±0.11
Isopenthylamine	4.46±0.41	N/A±N/A	4.213±0.071	9.66±0.35	18.85±0.64	1.53±0.59
Putrescine	1173±40	873±9	652±39	1617±31	969±11	785±22
Spermidine	91±26	33.93±0.31	105±2	86.4±1.5	94.1±4.2	150.3±2.5
Spermine	30.1±1.3	18.50±0.44	10.61±0.12	11.53±0.35	22.65±0.21	24.2±0.5
Tyramine	215±11	54.9±1.6	85.4±5.2	669±22	164±7	61.8±2.3
Tryptamine	33.5±1.9	26.23±1.08	19.1±1.4	27.57±0.93	24.50±0.28	13.10±0.36
Histamine	8.7±3.3	10.2±1.8	3.48±0.68	13.77±0.32	39±2	3.98±0.42
Hexsylamine	N/A	N/A	N/A	N/A	N/A	N/A
Agmatine	4000±300	1403±75	1727±12	1603±60	1260±0	840±47
Cadaverine	79±2	47±8	177.7±4.2	73.9±1.3	114.50±0.71	16±1

Compund	Schwarzbier 2	Lager 1	Non-alcoholic 2	Lager 2	Weizen 2	Lager 3
Dimethylamine	12.43±0.38	1.760±0.044	0.834±0.025	1.650±0.075	3.073±0.068	2.987±0.059
Methylamine	63.2±2.2	40.93±0.38	10.80±0.46	25.03±0.45	22.90±0.89	26±1
Ethylamine	2013±42	2173±81	556±35	1437±102	765.3±16.2	1153±35
Diethylamine	0.68±0.21	0.41±0.23	N/A	1.4±1.6	1.9±2.2	0.9±1.5
Propylamine	N/A	N/A	N/A	N/A	N/A	N/A
Butylamine	63.2±2.2	112.7±2.9	39.1±1.4	85.7±2.8	104.8±9.9	117.7±9.3
Isobuthylamine	27.2±1.2	5.75±0.82	N/A	3.86±0.62	4.8±0.7	1.797±0.092
Phenylethylamine	21.4±0.9	4.95±0.57	0.580±0.056	3.29±0.34	7.20±0.51	93±5
Isopenthylamine	34.1±1.4	7.74±0.53	2.37±0.18	4.96±0.46	6.89±0.34	4.69±0.14
Putrescine	814±14	1223.3±5.8	530.3±5.1	1007±25	1250±26	1270±36
Spermidine	108±4	160±1	142.3±7.5	105±12	122.7±1.2	313.3±3.2
Spermine	38.2±1.1	40.1±1.3	21.30±0.46	27.33±0.76	30±2	44.5±4.7
Tyramine	168.3±2.1	216.7±6.4	86±4	150.7±5.1	226±9	3723±35
Tryptamine	14.70±0.46	32.1±1.5	14.87±0.61	32.33±0.71	58.4±2.2	44.4±2.4
Histamine	15.3±1.5	21.4±1.2	3.18±0.27	11.9±0.4	51.2±2.2	22.7±1.5
Hexsylamine	N/A	N/A	N/A	N/A	N/A	N/A
Agmatine	2230±85	5290±187	1767±107	3323±65	2347±45	3437±31
Cadaverine	99.6±1.7	93.40±0.36	20.27±0.65	70.5±2.4	129.3±3.8	68±2

3.3 Application to real-world samples

The last step was the application of the method on beer samples analysis. Twelve different beers were analyzed and compared between each other. In non-alcoholic beer, level of biogenic amines was lower than in beers with alcohol (Fig. 2.). Content of 2-phenylethylamine in non-alcoholic beer is one hundred times smaller than in alcoholic beer (the same brand). It is worth to noting, that content of cadaverine is on the same level. What is more, in beer (Lager) the content of tyramine is the highest of among of all the biogenic amines. In the other hand, in case of non-alcoholic beer, content of tryramine is significantly lower.

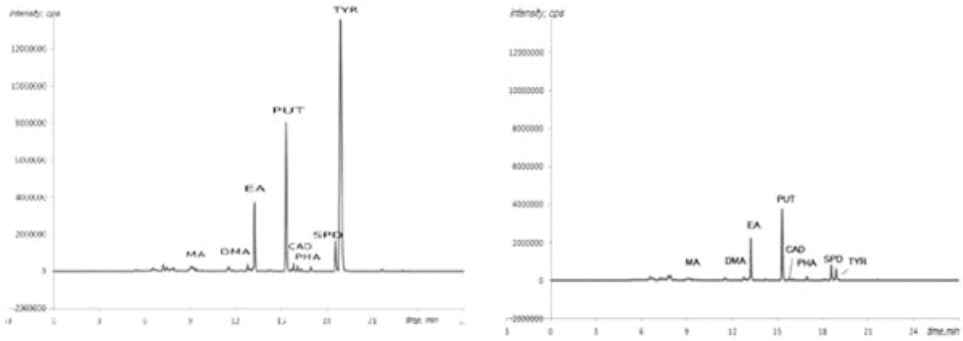

Fig. 2. HPLC-MS/MS chromatograms of analysed beers (A) Lager, (B) non-alcoholic beer. Biogenic amines: methylamine (MA), dimethylamine (DMA), ethylamine (EA), putrescine (PUT), cadaverine (CAD), phenylethylamine (PHA), spermidine (SPD), 2- tyramine (TYR).

4. Conclusion

An acurate and repeatable method to determination of eighteen biogenic amines in beers was developed. System HPLC characterized a high resolution, because of the good separation of 18 biogenic amines. What is more, method enables the analysis at low level of concentrations of biogenic amines. Futhermore, the method characterised satisfactory linearity (R^2>0.99), repeatability and high sensitivity. The developed method was used successfully for the determination of biogenic amines in different type of beers (Lager, Schwarzbier, Weizen, Non-alcoholic beers, Pils, Stout, Strong Lager). In case of diethylamine, method is not very repeatable, therefore further research on the method for determining biogenic amines in beer will be carried out.

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Analysis of novel members of FAHFA lipid class derived from omega 3-PUFA by LC-MS/MS and LC-MS/MS/MS

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Abstract
White adipose tissue (WAT) is a complex organ with both metabolic and endocrine functions. Dysregulation of all of these functions of WAT, together with low-grade inflammation of the tissue in obesity, contributes to the development of insulin resistance and type 2 diabetes. Omega-3 polyunsaturated fatty acids (PUFA) of marine origin play an important role in resolution of inflammation and exert beneficial metabolic effects. Using LC-MS/MS method, we elucidated the structures of novel members of fatty acid esters of hydroxy fatty acids (FAHFA), which were present in murine and human serum and WAT after omega-3 PUFA supplementation in diet. These compounds contained DHA esterified to 9- and 13-hydroxyoctadecadienoic acid (HLA) termed 9-DHAHLA and 13-DHAHLA, and were synthesized in WAT. Our results document the existence of novel lipid mediators, which are involved in the beneficial anti-inflammatory effects attributed to omega-3 PUFA, in both mice and humans.

1.Introduction

White adipose tissue (WAT) is an extremely plastic organ with important roles in energy balance, whole body glucose homeostasis and the immune system [1]. Adipocytes in WAT also release lipid-based mediators such as branched fatty acid hydroxy fatty acid esters (FAHFAs) [2], which could improve local and whole body glucose metabolism [3]. Moreover, FAHFA administration also reduced obesity-associated WAT inflammation in mice [2].

The FAHFA nomenclature introduced by Yore et al. [2] combines abbreviations of esterified FAs and hydroxy FAs, e.g. the combination of esterified palmitic acid (PA) and hydroxyl stearic acid (HSA) was abbreviated as PAHSA. Although a recently published in-silico library of all potential FAHFAs uses a nomenclature based on chemical structure [4], for practical reasons, here we use the shorter abbreviations, e.g. PA for palmitic acid, LA for linoleic acid (LA), DHA for docosahexaenoic acid with the "H" prefix for hydroxy fatty acids and the position of branching.

Given the beneficial effects of omega-3 PUFA on WAT inflammatory status, we hypothesized that novel FAHFA structures derived from omega-3 PUFA, with possible anti-inflammatory properties, could be found. To test this hypothesis, we performed lipidomic analysis using human and murine serum and WAT samples collected from subjects supplemented or not with omega-3 PUFA.

2.Experimental

2.1 Reagents and chemicals

All chemicals were purchased from Sigma-Aldrich. FAHFA standards were purchased from Cayman Pharma (Neratovice, Czech Republic). Organic synthesis of 13-DHAHLA was performed according to Steglich esterification from DHA and 13-HODE [5]. Details are provided in [6].

2.2 Human and mice samples and cell lines

Human serum samples were acquired within the framework of a clinical trial [7]. Briefly, patients diagnosed with type 2 diabetes, were given either 5 g/day corn oil (Placebo) or 5 g/day EPA+DHA concentrate (Omega-3; EPAX 1050TG, EPAX AS, containing about 15% EPA, 40% DHA, wt/wt; i.e., ~2.8 g EPA+DHA) for 24 weeks. Afterwards, the serum samples and biopsies of abdominal subcutaneous WAT were collected. Male mice (C57BL/6J; Jackson Laboratory, ME, USA) were fed a corn oil-based high-fat diet (HF; lipid content 35%, *wt/wt*) or HF diet with EPA+DHA (HFF) concentrate (EPAX 1050TG) for 8 weeks as before [8]. Epididymal WAT, subcutaneous WAT, liver and interscapular brown adipose tissue and serum samples were collected. Murine adipocyte cell line 3T3-L1 was grown according to standard protocols [2]. Differentiated adipocytes were incubated with 100 μM LA and 100 μM DHA complexed to BSA 3:1 for 24 hours and extracted for FAHFA analysis. RAW 264.7 cells and murine bone marrow-derived macrophages (BMDM) were grown and stimulated with lipopolysaccharide (LPS) as before [9].

2.3 FAHFA extraction and liquid chromatography-mass spectrometry

FAHFA extraction was performed based on the published method [2]. Chromatographic separation was performed at UPLC Ultimate 3000 RSLC (Thermo)

equipped with a Kinetex C18 1.7 μm 150×2.1 mm column (Phenomenex). The flow rate was 200 μl/min at 50 °C. The mobile phase consists of solvent A (70% water, 30% acetonitrile, 0.01% acetic acid, pH = 4) and solvent B (50% acetonitrile, 50% isopropanol). We used a gradient elution for separation of FAHFAs whereas isocratic elution (20% A, 80% B, 60 minutes) was used for structural studies. FAHFA were detected in negative ESI mode. Multiple Reaction Monitoring (MRM) mode with one quantifier and two qualifier transitions per FAHFA was used for quantitation. Quantifier ion MRM was used as a survey scan for information-dependent acquisition in the linear ion trap for enhanced-resolution MS/MS and MS/MS/MS spectra.

2.5 Markers of inflammation

Murine IL-6 ELISA (Cayman Chemicals) and qPCR [8] were used to measure the markers of macrophage activation. BMDM were incubated in the presence of LPS alone or in combination with DHA (10 μM), IFN-γ (50 ng/ml) or 13-DHAHLA (10 μM) for 18 hours. LC-MS/MS metabolipidomics [9, 10] was used to measure macrophage metabolic activation and levels of lipid mediators.

3.Results and discussion

3.1 Identification of novel FAHFA derived from LA and DHA

We developed a targeted lipidomic methodology using liquid chromatography coupled to hybrid tandem mass & linear ion trap spectrometry to identify and quantify FAHFAs in human and murine samples. We took advantage of the ability of MS to switch from sensitive triple quadrupole scan modes to highly sensitive full-scan ion trap mode within one analysis to obtain both quantitative and qualitative (structural) information. This approach enabled us to precisely quantify FAHFA levels using MRM and identify the branching position on the backbone HFA using MS/MS/MS.

Using this MS approach we were able to identify novel members of the FAHFA family derived from LA and DHA, specifically DHAHLA in human serum. With DHAHLA, DHA esterified to a hydroxy LA, two positional isomers of the hydroxy FA backbone were detected: 9- and 13-hydroxyoctadecadienoic acid (HLA aka HODE), therefore 9- and 13-DHAHLA. As shown in the 13-DHAHLA fragmentation scheme (Fig. 1A), the ion 605.457 *m/z* gave rise to the fragment 295.228 *m/z* (13-HODE), which was further fragmented to characteristic ions 179.144 and 195.139 *m/z*. Fig. 1B provides MS/MS spectrum of DHAHLA.

Chromatographic separation of the murine serum sample revealed additional complexity of DHAHLA isomers when four separated DHAHLA peaks were detected (Fig. 1C). Structural characterization in the linear ion trap revealed that two major peaks were 13-DHAHLA and 2 minor peaks were 9-DHAHLA *cis*-trans

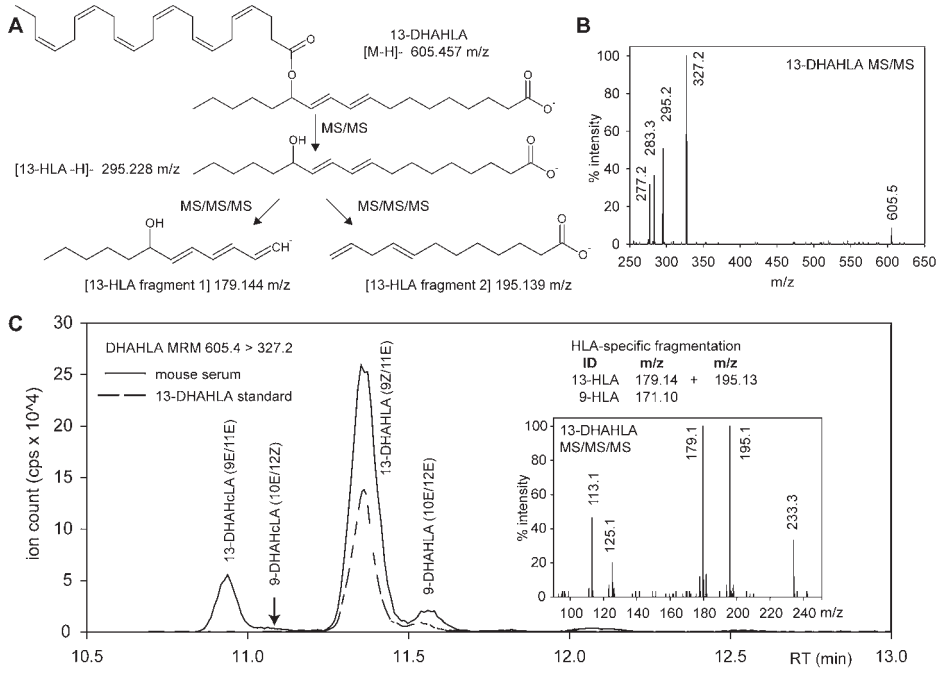

Fig. 1. Analysis of DHAHLA isomers: (A) fragmentation scheme of 13-DHAHLA with 13-HLA-specific fragments. (B) MS/MS spectrum of 13-DHAHLA. (C) Chromatographic profile of DHAHLA isomers (MRM 605.4 > 327.2) detected in murine serum sample (solid line) overlaid with synthetic standard of 13-DHAHLA (dashed line). Inserted table summarizing MS/MS/MS fragments specific to individual positional isomers of the hydroxyl group on HLA and MS/MS/MS spectra of 13-DHAHLA. Specific fragments highlighted in magenta. HcLA, hydroxy-conjugated-LA. The figure is adapted from Kuda et al. [6] with authors permission.

isomers of double bonds in HLA acyl chains. Identity of the backbone fragmentation was confirmed using synthetic standards for 9(S)-HODE and 13(S)-HODE (Fig. 1C).

13-DHAHLA was detected in murine adipose tissue depots and was upregulated after omega-3 PUFA supplementation. Very low levels of 13-DHAHLA were detected in human subcutaneous fat biopsies after omega-3 PUFA supplementation (0.38±0.06 pmol/g), but no DHAHLA was detected in placebo-treated patients. The levels of DHAHLA were comparable to the concentrations of DHA-derived docosanoids (protectins, resolvins) in human serum. Cultured 3T3-L1 adipocytes, when supplemented with DHA and LA, were able to synthesize DHAHLA isomers.

3.2 Anti-inflammatory and pro-resolving effects of DHAHLA

Reflecting the anti-inflammatory effects of PAHSAs, the already known members of FAHFA lipid class, on adipose tissue macrophages from obese mice [2] and the

beneficial effects of DHA and its metabolites on adipose tissue inflammation [10], we hypothesized that DHAHLA could also have immunomodulatory properties. First, RAW macrophages were stimulated with LPS, and the effects of 9-PAHSA [2] and 13-DHAHLA on macrophage activation were analyzed. Both PAHSA and DHAHLA prevented the increase of pro-inflammatory IL-6 concentrations in media and also decreased mRNA levels of Il-6, Tnf-α, Il-1beta and Ptgs2 in cells.

Next, BMDM were incubated in the absence (unstimulated) or presence of IFN-γ (50 ng/ml) for 18 hours and the effect of 13-DHAHLA (10 μM) on macrophage activation (intracellular citrulline levels) was tested. 13-DHAHLA prevented macrophage activation. These results document that 13-DHAHLA itself exerts anti-inflammatory and pro-resolving properties.

4. Conclusion

To conclude, we identified novel members of FAHFA lipid class derived from DHA and LA (e.g. DHAHLA) with anti-inflammatory properties in the serum and WAT of both mice and diabetic patients supplemented with omega-3 PUFA by our lipidomic methodology using LC-MS/MS. Our results suggest the involvement of the novel lipids in the broad beneficial effects of omega-3 PUFA on health. As demonstrated by our clinical trial [7], omega-3 PUFA could improve postprandial lipid metabolism in overweight/obese patients with type 2 diabetes, even in the face of a combined pharmacotherapy.

Acknowledgments

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Identification of aromatic compounds in odours mixture by gas chromatography and field olfactometry techniques

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Abstract
This paper shows the results of investigation on identification aromatic compounds present in odorous mixture in atmospheric air samples collected in a vicinity of the oil refinery LOTOS S.A., located in Gdansk, during March and April period have be presented. The studies were conducted by the use of comprehensive two-dimensional gas chromatograph equipped with a cryogenic modulator and coupled with a time-of-flight mass spectrometer, and Nasal Ranger field olfactometer. The use of these techniques allows to characterize sensory properties of aromatic compounds present in odours mixture in the air.

1. Introduction

Quality of ambient air is depending on all forms of human activity, such as degree of urbanization of the areas and deployment of data-area industrial plants or municipal facilities (municipal waste landfill sewage treatment plants) [1–3]. Dynamic economic and industrial development contributes to increased amount of pollutants. These compounds can have a negative effect on living organism as well as abiotic part of environment. Some of them have also carcinogenic properties. Among numerous pollutants present in the atmospheric air, particular attention should be directed to all kinds of malodorous substances – odorants [4]. Compounds responsible for unpleasant aroma, both aliphatic and aromatic (hydrocarbons, amines, aldehydes, ketones, carboxylic acids, esters) all belong to this group. Their heterogeneity makes them a particularly difficult research subject [5, 6].

Measurement techniques used to determine the level of pollution emission from various sources, can be divided into two basic groups: instrumental, and

Fig. 1. Location of measurement points.

sensory techniques. Gas chromatography coupled with mass spectrometry detector (GC-MS) is commonly used instrumental techniques for quantitative and qualitative determination of chemical compounds present in air [7, 8]. Among sensory techniques, field olfactometry enjoying an increasing popularity in view of ability to determination of odorous concentration directly in emission sources [9–11].

In this work research concerning identification aromatic compounds present in odorous mixture using gas chromatography and field olfactometry techniques. The subject of the study was atmospheric air samples collected around oil refinery LOTOS S.A., located in Gdansk. This research can show, which from the aromatic compounds are present in odorous mixture and can have a significant impact of odorous nuisance level.

2. Experimental

2.1 Sampling

Fig. 1 presents locations of measurement point, located around the LOTOS S.A. oil refinery, one of the biggest industrial facilities in Nasal Ranger Pomerania Voivodeship. At points P1–P5 sensory evaluation of the air using Nasala Ranger field olfactometer was conducted and samples for chromatographic analysis were collected. At each measurement point, air pollutants were adsorbed on poly-(2,6-diphenyl-*p*-phenylene oxide) known by its trademark Tenax TA using a Gas

Sampling System (GSS) manufactured by GERSTEL, Germany. The measurements were conducted in a three-week period in March and April 2016.

2.2 Instrumentation

Air samples were analysed using a two-dimensional gas chromatograph equipped with a cryogenic modulator and coupled with a time-of-flight mass spectrometer. The column set consisted of a 30 m × 0.25 mm × 0.25µm primary column (1D) with Equity 1 stationary phase (Supelco) and a 2.0 m × 0.10 mm × 0.10 µm secondary column (2D) with Sol-Gel-Wax stationary phase (SGE Analytical Science). The sample components were separated using the following optimized temperature program for *(i)* the primary GC oven: initial temperature of 40 °C maintained for 1min, ramped at 10 °C/min to 90°C, ramped at 3 °C/min to 240°C, kept temperature for 5min; and for *(ii)* the secondary GC oven: initial temperature of 45 °C maintained for 1min, ramped at 10 °C/min to 95°C, ramped at 3 °C/min to 245°C, kept temperature for 5min. As a carrier gas, *h*elium was used at a constant flow of 1.0 ml/min. The temperatures for the transfer line and ion source were maintained at 250°C. The detector voltage was set to 1600V. Ions in the *m/z* 40–500 range were analyzed.

Sensory analysis was conducted with Nasal Ranger field olfactometer (St. Croix Sensory, USA). During measurements with field olfactometer, each member of the four-person team of panelists that evaluated the odour was asked to indicate at what concentration (beginning from the greatest dilution) the odour became perceptible. Each panellist conducted 3 evaluations in each location in 10-minute intervals.

3. Results and discussion

3.1. Sensory analysis

Odours concentration was calculated according to the following equation:

$$Z_{\mathrm{ITE}} = \sqrt{Z_{\mathrm{YES}} \cdot Z_{\mathrm{NO}}} \tag{1}$$

where: Z_{ITE} is individual odour detection threshold, Z_{YES} is dilution corresponding to the *D/T* value at which the odour is first perceived, and Z_{NO} is dilution corresponding to the *D/T* value at which the odour is not yet perceptible. Table 1 shows a concentrations of odours from each measurement point. It can be observed in location P2 and P3 the values of concentrations of odorants present in atmospheric air samples were higher than others. These two points are located close proximity of the sewage treatment plant Gdańsk-East, so it could be a significant factor of higher concentration of odours in location P2 and P3. A concentration of malodorous substances at each measurement point is also largely due to atmospheric conditions, in particular wind speed, temperature and direction.

Table 1
Concentration of odorants at each measurement point [ou/m^3].

Location	1-st person	2-nd person	3-rd person	4-th person	Mean value
P1	1.7	1.7	1.7	1.7	1.7
P2	5.8	4.5	4.6	4.8	4.9
P3	6.3	9.3	9.0	7.9	8.1
P4	1.7	1.7	1.7	1.7	1.7
P5	1.7	1.7	1.7	1.7	1.7

Table 2
Various chemical compounds identified using gas chromatography technique at location P2 and P3.

Location P2		Location P3	
Compound	*S/N*	Compound	*S/N*
Benzene	70154	Benzaldehyde	2773
Tetradecane	21424	2-Undecanone	1544
Hexane	18575	Benzene	1316
D-Limonene	18569	Nonane	663
Pentane	14821	Decanal	615
Benzene, 1,3-dimethyl-	13857	Pentadecane	466
α-Pinene	9702	1-Nonene	421
Acetaldehyde	7008	Eicosane	420
Sabinene	6517	Octanal	359
Sabinene	6517	Tridecane	358
o-Xylene	5061	Hexadecane	283
1-Hexene	5049	3-Hexanone, 5-methyl-	282
Benzaldehyde	4171	2-Dodecanone	282
Dodecane	3524	Heptanal	278
Pentadecane	2781	Benzene, butyl-	249
Cetene	2677	1-Tetradecanol	248
Ethanone, 1-phenyl-	2209	1-Tetradecene	220
γ-Terpinene	1533	Hexanal	169
Hexadecanoic acid	1422	Dibutyl phthalate	129
Nonanal	1353	Biphenyl	45

3.2. Gas chromatography analysis

Using gas chromatography technique, it was possible to identify the most significant pollutants present in atmospheric air at each measurement point. Table 2 shows chemical compounds present in P2 and P3 location (where odours concentrations were highest), characterised by the greatest signal/noise (S/N) ratio. Among the chemical compounds listed in Table 2, there are aromatic compounds such as: benzene, benzaldehyde, *o*-xylene, butylbenzene, dibutyl phthalate, biphenyl, and 1,3-dimethylbenzene. Some of these compounds can

have a responsible for malodour. Their impact on odour nuisance in varying degree, since they are characterised by different odour properties: odour intensity, hedonic quality, concentration of each compounds in air, or type of smell malodorous substances. Among the identified aromatic chemical compounds, benzaldehyde has characteristic almond-like odour, *o*-xylene is a sweet-smelling liquid, and biphenyl has a distinctively pleasant smell. In order to verify, which from these compounds can have significant impact on the odour nuisance level in the areas adjacent to the LOTOS S.A. oil refinery, a quantitative analysis would have to be performed and compare these results with individual odour detection threshold.

4. Conclusions

In the research presented in this article instrumental solution – two dimensional gas chromatography with mass spectrometry technique was used, and also a sensory analysis using Nasal Ranger field olfactometers was performed. The application of GC-GC-TOF-MS facilitated identification of aromatic compounds present in air sample in location, in which concentration of odours were the highest from each measurement locations. Aromatic compounds can have impact of odour nuisance level, but it is depend on the various factors, such as aroma properties or air condition. Some of these compounds have very pleasant smell but combination malodorous substances in various concentration can give unpleasant olfactory sensation.

Acknowledgments

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The impact of addition of olive oil on thermal degradation of refined rapeseed oil

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Keywords
edible oils
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ultra-fast gas chromatography

Abstract
Fats are an important component of the everyday diet and have a significant impact on the proper functioning of human organism. However, during the process of frying chemical transformations take place in the oil; hence fats characterised by high oxidative stability should be given preference. The aim of this work was to determine the quality of rapeseed oil, blended oil, and refined olive oil, all sourced from the domestic market. Oxidative stability of oils was determined using the Rancimat test, and the degree of thermal degradation was estimated using ultra-fast gas chromatography. Based on the obtained results it was concluded, that the addition of olive oil improves the quality of rapeseed oil. Blended oil displays higher oxidative stability and a lower degree of thermal degradation as compared to rapeseed oil.

1. Introduction

Frying is one of the oldest methods of food processing. It is based on thermal treatment of food dipped in animal fat or oil of botanical origin [1]. The temperature of this process is 170–190 °C. During frying chemical transformations take place in the oil, such as oxidation, hydrolysis, cyclisation, or polymerization [2–5]. The products of thermal degradation of vegetable oils are, on the one hand, responsible for desirable flavour and aroma of prepared dishes, but on the other hand may be hazardous to human health. Products of thermal decomposition of oils such as polycyclic aromatic hydrocarbons, or some aldehydes may be the cause of cancer, diabetes, and atherosclerosis [3]. One of the oils most commonly used for frying is rapeseed oil. The reasons are a relatively low price, material availability, and good frying parameters [6]. However, refined olive oil is thermally more stable, and also is more flavourful [7]. Recently, blended vegetable oils comprised of rapeseed oil and olive oil, have become increasingly popular. Usually, olive oil constitutes no more than app. 5% of the whole product. Its addition

improves the palatability and functional properties of the blended oil. The reference methods used to determine the quality of oil are the measurement of the Totox parameter, determination of oxidative stability using the Rancimat test, or determination of free fatty acids using gas chromatography [8]. These methods are time-consuming and labour-intensive, and sometimes require sample preparation. In this work a method of evaluation of thermal degradation of selected vegetable oils based on the headspace characteristic using ultra-fast gas chromatography is proposed. The described technique does not require sample derivatisation and preparation and is characterized by a very short time of a single analysis.

2. Experimental

2.1 Reagents and chemicals

Vegetable oils were obtained at local distribution centres in Gdańsk. Samples were refined oils, i.e. rapeseed oil, olive oil, and commercially available blended oil (a mixture of rapeseed oil and olive oil; 5% *v/v* addition of olive oil). A sample of 5 grams of each vegetable oil was poured into 20 ml glass vials that were then sealed with a cap with a silicone-PTFE membrane. Thus prepared samples were heated for 24 hours at 20 °C, 60 °C, 100 °C, 140 °C, and 180 °C. In the analytical procedure, in which the oxidative stability of edible oils was determined, deionized water of high purity obtained using the MilliQ A10 device (Millipore) was used.

2.2 Instrumentation

In order to determine the oxidative stability of edible oils the 893 Professional Biodiesel Rancimat (Metrohm) was used. In accordance with ISO 6886:2006 during the measurement process samples were kept at 120 °C. The volumetric air flow was set at 20 L/h. Obtained data was processed using the StabNet software. The analysis of the volatile fraction of vegetable oils was conducted using ultra-fast chromatography device Heracles II equipped with the HS 100 autosampler (Alpha M.O.S.) was used. Data analysis was performed using AlphaSoft v. 12.4 software.

3. Results and discussion

The reference method for the evaluation of edible oils quality is the measurement of oxidative stability. In order to determine this parameter tests of accelerated oxidation are used, e.g. the Rancimat test. Oxidative stability is measured based on the induction time, that is the time after which the oil oxidizes at a given temperature. Superior quality oils are characterized by longer induction times. Depicted

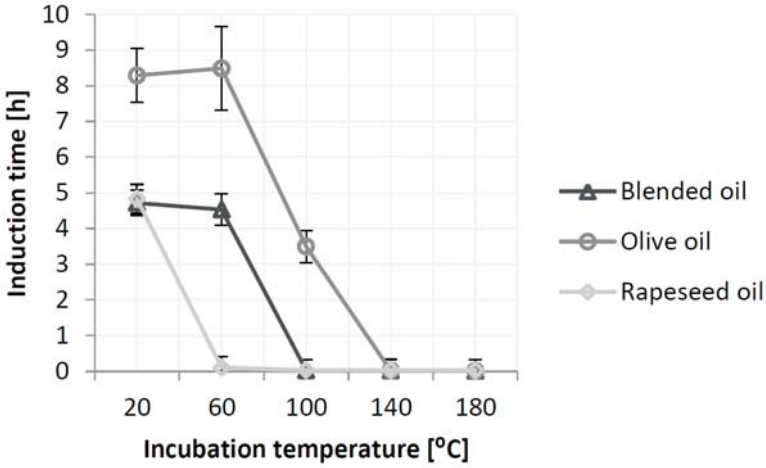

Fig. 1. Oxidative stability of rapeseed oil, blended oil and olive oil as a function of sample incubation temperature.

in Fig. 1 is the function of induction time and incubation time of samples. With the rise of incubation time, the oxidative stability decreases. Olive oil is the most stable. The oil that oxidizes the fastest is rapeseed oil. This is due to the fact, that rapeseed oil contains greater quantities of polyunsaturated fatty acids, which are susceptible to oxidation [9]. The presence of 5% olive oil adjunct in the blended oil increases its thermal stability. The induction time of rapeseed oil incubated at 60 °C was close to zero, which means, that this oil oxidized the fastest. In the case of blended oil, as well as olive oil a sharp decrease of induction time at incubation temperature of 100 °C may be observed. These oils display a lower degree of thermal degradation.

The use of ultra-fast gas chromatography enables a qualitative analysis of oil sample's headspace. In Table 1 is presented the composition of volatile fraction of a blended oil incubated at 180 °C. Kovats indices for both chromatography columns (MXT-5, MXT-1701) are given for each compound. Aroma descriptors are taken from the AroChemBase database that is a part of AlphaSoft v. 12.4 software.

Based on the results obtained during the headspace analysis the degree of thermal degradation of edible oils was estimated. To this end, statistical quality control model (SQC) was used. SQC can be utilized to indicate the differences in the chemical composition of the sample's headspace. During the research it was decided, that samples of vegetable oils incubated for 24 h at 20 °C will be used as reference. The diagram, based on which the evaluation of the degree of thermal degradation of blended oil and olive oil was conducted is presented in Fig. 2. Based on the results it may be stated, that thermal degradation of blended oil and olive oil samples incubated at 60 °C was relatively low. The samples of rapeseed oil

Table 1
Qualitative analysis of blended oil samples incubated for 24 h at 180 °C using ultra-fast gas chromatography.

Compound	Kovats index (MXT-5)	Kovats index (MXT-1701)	Aroma description
Butane	400	400	–
Methanol	419	507	pungent
2-Methyl-2-propanol	494	621	–
1-Propanol	525	661	alcoholic, fruity, musty, plastic
tert-Butylmethylether	546	600	–
Butanal	578	660	chocolate, green, malty, pungent
Hexane	600	600	alkane, ethernal, kerosene
Ethyl acetate	609	673	butter, caramelized, fruity, ethernal, orange, pungent, sweet
But-(E)-2-enal	646	751	floral, green, plastic
1-Penten-3-one	682	765	fishy, fruity, rotten, spicy
2-Ethyl furan	703	735	rubber, sweet
Propyl acetate	708	775	caramelized, fermented, fruity, sweet
Methyl butanoate	717	784	fruity, green, sweet
(E)-3-Penten-2-one	735	836	–
2-Methylpentanal	754	837	earthy, fruity
(E)-2-Penten-1-ol	769	888	grassy, green, mushroom
1-Hexen-3-ol	775	880	green
Butyl acetate	810	879	banana, bitter, fruity, green, sweaty
(E)-2-Octene	815	819	–
Ethylbenzene	875	918	ethernal, floral, sweet
2-Butylfuran	893	927	spicy
(E,E)-2,4-Hexadienal	912	1050	green, vegetable
Methyl hexanoate	925	988	fresh, fruity, thinner
Benzaldehyde	959	1086	almond, fruity, woody
2-Heptenal, (E)-	960	1062	almond, earthy, grassy, onion
Phenol	979	1219	medical, phenolic
Benzyl alcohol	1034	1220	floral, fruity
(Z)-2-Octenal	1045	1157	earthy, leafy, walnut
3,5-Octadien-2-one	1092	1193	fatty, fruity, mushroom
Nonan-2-one	1093	1183	baked, earthy, fatty, fruity
E-2-Nonen-1-ol	1173	1185	green
Tridecane	1300	1300	fusel, citrus, fruity
Skatole	1393	1634	animal, fecal,
Pentadecane	1500	1500	mild green

incubated at this temperature displayed a higher degree of degradation. The highest degree of thermal degradation was observed for rapeseed oil. It was also demonstrated, that the amount of the products of thermal degradation (that is polar organic compounds) was higher in the case of rapeseed oil samples. The results obtained for blended oil samples confirm the hypothesis, that the addition of olive oil lowers the degree of thermal degradation of oil.

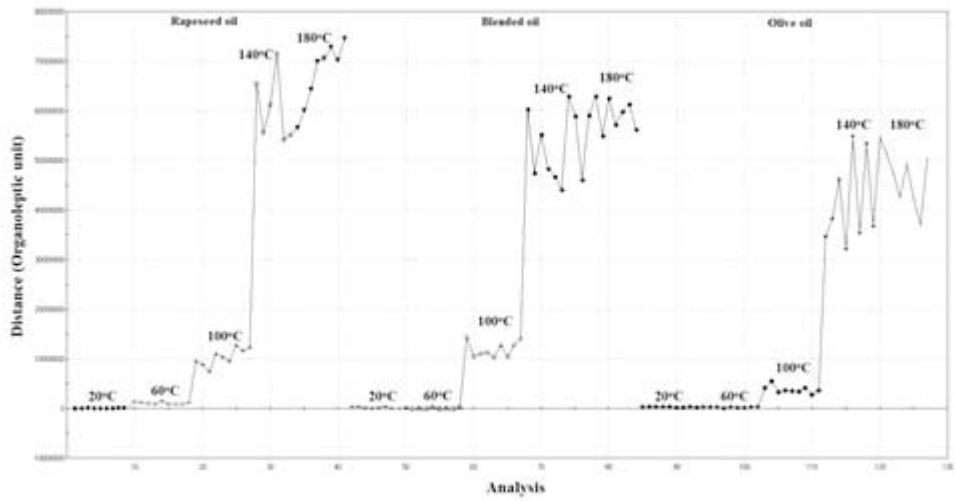

Fig. 2. The relationship between the degree of thermal degradation and temperature at which the samples of rapeseed oil, blended oil and olive oil were incubated.

4. Conclusions

Based on the obtained results a hypothesis can be made, that the addition of olive oil improves the characteristic of rapeseed oil. A blended oil displays higher oxidative stability and a lower degree of thermal degradation. The employed method of evaluation of edible oil's quality using ultrafast gas chromatography is characterized by a relatively short time of a single analysis and no sample preparation. It is the author's opinion, that in the near future it will be possible to use the described method for evaluation of the degree of thermal degradation of vegetable oils as complementary to existing methods of determining the quality of edible oils.

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Meat freshness classification using ultra-fast gas chromatography

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Keywords
food analysis
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ultra-fast gas chromatography

Abstract
Meat constitutes an important component of human diet. In order to ensure that it is safe to consume, it is important to be able to determine whether a particular sample is fresh. To this end, an analysis of the headspace of pork, beef, and poultry was performed over a period of seven days using ultra-fast gas chromatography. Compounds that may possibly be used as indicators of spoilage were identified. Based on the obtained results it was concluded, that it is possible to distinguish between particular meat samples based on the day of analysis, as well as differentiate between different kinds of meat.

1. Introduction

Animal meat is one of the basic food products. It is an important element of many diets due to a high content of easily digestible protein, as well as highly caloric fat and vitamins and microelements necessary for maintaining proper metabolic processes. For that reason, and also because of the constant increase of the global population, the market of meat and meat products is continuously growing. In the sector of meat production and processing there are numerous challenges pertaining to the evaluation of meat quality, both at the industrial [1] and retail level [2]. The main factors that are taken under consideration by consumers when choosing a meat product are colour and aroma. However, simple sensory evaluation of hedonistic qualities is often not enough to determine, with reasonable certainty, weather the protein in question is spoiled or not. To this end, a device equipped with a chemical sensor, sensitive to a compound the presence of which indicates spoilage, would prove useful. Unfortunately, no single compound has yet been identified as the one primarily responsible for the aroma of meat [3]. On the other hand, a combination of volatile compounds may form a unique "fingerprint", an aroma profile, which may be used as an indicator of spoilage or to differentiate between types of meat. To that aim devices called electronic noses may be used with good results [4–6].

2. Experimental

2.1 Reagents and chemicals

Beef, pork and, poultry samples were obtained in the local distribution centres in Gdańsk, Poland. Samples of 3 g were placed in a 20 cm^3 vial and sealed with a cap lined with a silicon-PTFE membrane. The samples were analysed over the period of seven consecutive days, with 12 samples analysed every day – four of each type of meat. Prior to analysis the meat was refrigerated at 7 °C.

2.2 Instrumentation

Headspace analysis of meat samples was performed using an ultra-fast gas chromatography unit Heracles II equipped with the HS100 autosampler (Alpha M.O.S.). The device was equipped with two 10 m columns packed with MXT-5 and MXT-1701 stationary phases, respectively. AlphaSoft 12.4 software was used to process the data. Data analysis was performed using principal component analysis (PCA) and statistical quality control (SQC).

3. Results and discussion

The aim of the research was to determine, weather it is possible to discriminate between meat samples stored over the period of seven days using multidimensional data analysis and, in particular, weather it is possible to indicate that the sample is no longer fresh based on its headspace. The changes in the aromatic profile were monitored using an ultra-fast GC device working, in this instance, as an electronic nose. The chromatographic peaks from both flame ionisation detectors (FID) were treated as signals of virtual gas sensors. Data processing, namely SQC method was later used to verify the hypothesis that at some point over the span of research the profile of the meat's headspace would change significantly, which would indicate spoilage. The assumption proved to be true. By employing data analysis it was possible to distinguish two separate classes of profiles, namely fresh and spoiled. In the case of pork and beef the change between the two classes took place after the second day of storage, which confirms previously reported results [7]. In the case of poultry the change took place after the third day. An example of SQC analysis output is presented in Fig.1. The classes were clearly separated, with all the data points before the presumed spoilage included in the confidence envelope, with an exception of a single outlier in case of pork. Additionally, a PCA analysis was performed in order to determine, weather it is possible to clearly distinguish between the three types of meat at each day of the experiment, which yielded positive results. Data points corresponding to samples of beef, pork and poultry formed distinct and separate groups at each of the seven days of testing. A sample PCA biplot for the last day of the experiment is presented in Fig.2.

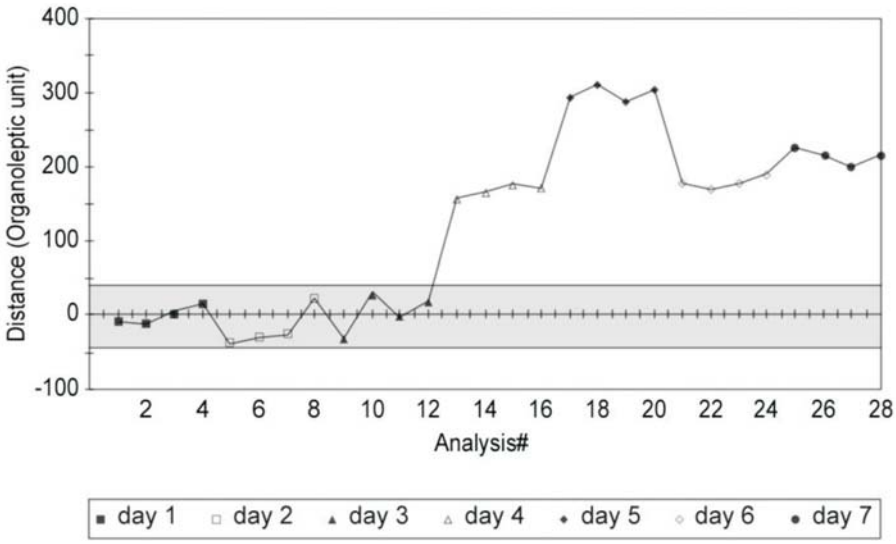

Fig. 1. Statistical quality control analysis of poultry samples over a period of 7 days.

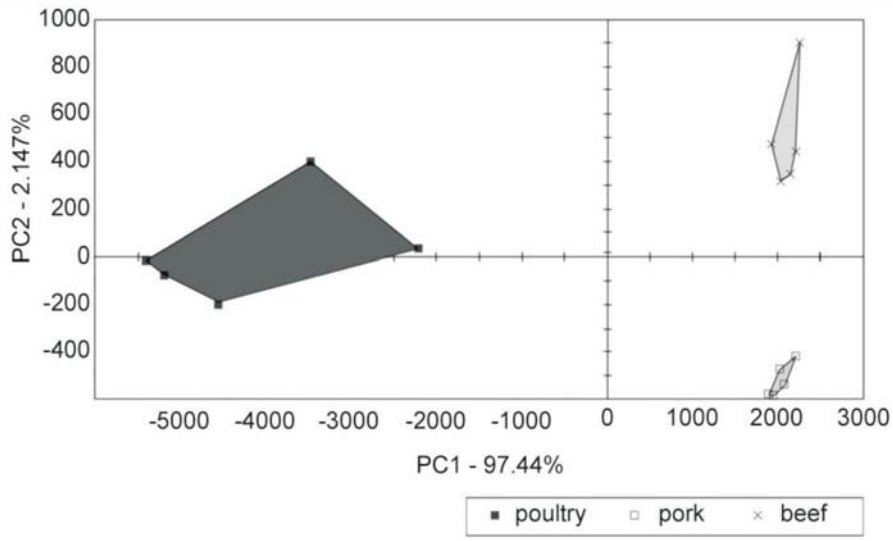

Fig. 2. Principal component analysis plot for samples of poultry, pork and beef at day 7**.**

The use of ultra-fast GC allows also for qualitative identification of volatile compounds present in the sample's headspace. The five compounds, the presence of which had the greatest impact on the statistical analysis are given in Table 1. It is important to note, that these compounds are not necessarily the ones with the highest concentration in the samples, but the ones for which the corresponding chromatographic peaks display the greatest variance over the period of seven

Table 1
Some compounds identified as possible meat spoilage indicators.

Compound	Sensory descriptor	Meat	Kovats index MXT-5/MXT-1701	Odour threshold [ppm]
Butan-2-one	Butter, cheese, chemical	Poultry, pork	587/685	0.74 [8]
Ethyl acetate	Acidic, butter, pungent, fruity	Poultry, beef	609/673	3.9 [9]
Heptane	Alkane, fruity, sweet	Poultry, beef, pork	700/700	200 [8]
3-methyl-1-butanol	Balsamic, bitter, burnt, fermented	Poultry, beef, pork	728/842	25 [10]
2,4-octadiene	Glue, warm	Poultry, beef, pork	816/825	–

days. These compounds can potentially be considered as universal indicators of meat spoilage, since the majority of them are present in all 3 meat types examined. Also given in Table 1 are Kovats indices for both chromatography columns (MXT-5, MXT-1701), sensory descriptors sourced from the AroChemBase database and odour thresholds.

4. Conclusions

Based on the obtained results it can be concluded, that it is possible to distinguish between particular meat samples based on degree of decomposition. The use of SQC method could be used to determine the expiry date of meat products, and also determine whether a sample is past that date. This data, combined with the identification of potential spoilage indicators, may prove a starting point of further research aimed at developing a system, that reliably indicates whether a given meat sample is safe to consume.

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Effect of substrate hydrophobicity on the electrocatalytic behaviour of phthalocyanine

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phthalocyanine
spectroelectrochemistry

Abstract
In this work, the electrocatalytic activity of pyridinoporphyrazine mediator deposited on two substrates with different hydrophobicity has been presented. We focused on hydrophobic highly oriented pyrolytic graphite (HOPG) as well as hydrophilic annealed gold electrode (Au111). The immobilization of mediator has been carried out spontaneous adsorption from aqueous solution on electrode surfaces. The different hydrophobicity surface resulted in change the electrocatalytic activities of mediator to hydrogen in aqueous solution. For this purpose, electrochemical, *in situ* spectroelectrochemical, and *ex situ* microscopically characterizations have been performed. The detailed comparison of surface morphology, surface coverage, surface roughness parameters, thickness of deposited layers of mediator, and pH solution has been studied.

1. Introduction

Porphyrazines are synthetic analogues of the more commonly investigated phthalocyanine complexes. These macrocycles have been extensively studied for many potential applications and are known to lower overpotentials in many electrochemical reactions. In addition they are commercially available, much cheaper than precious metallic catalysts, and it is possible easily changes their properties by the substituting the ring or replacement of the central metal.

Phthalocyanines have a high tendency to aggregate particularly in aqueous solutions due to their extended π-systems, thereby significantly reduce the active sites, and affect the catalytic activity [1].

It is known, that phthalocyanines deposited onto electrodes show better catalytic activity than when are used as homogeneous in solution. These compounds are able to form films on different types of electrode substrates, for example: gold [2], carbonaceous materials such as carbon paste [3], glassy carbon [4], highly oriented pyrolytic graphite [5], and carbon nanotubes [6]. The

immobilization of the active redox mediator can be achieved in various ways such as potential cycling methods [7], drop casting [8], spin coating [9], and mixing with carbon paste [10]. Significant characteristics to prepare films are morphology and thickness of active layers.

In this work, we describe how hydrophobicity of the use electrode substrate affects electrocatalytic activity of water-soluble porphyrazine mediator. We have studied the adsorption behaviour of water soluble non-aggregation *N,N´,N´´,N´´´*--tetramethyltetra-3,4-pyridinoporphyrazinocobalt, where thickness and morphology have been controlled by amount of active mediator and deposition time. Hydrogen was selected as model analytes and the electrocatalytic activity of film to hydrogen in aqueous solution at laboratory temperature has been investigated. The information obtained in this study is aimed at providing a more complete picture of the water soluble phthalocyanine assembly on both hydrophilic and hydrophobic surfaces and investigating whether water soluble phthalocyanine can be used to hydrogen sensing.

2. Experimental

2.1 Reagents and chemicals

The Cobalt(II) *N,N´,N´´,N´´´*-Tetramethyl-tetra-3,4-pyridinoporphyrazine 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 according to the literature [11]. All other chemicals were analytical grade and used without further purification. The $NaH_2PO_4.H_2O$, $Na_2HPO_4.2H_2O$ (p.a., Merck), H_3PO_4, NaOH (all p.a., Lachema, Czech Republic) were used for the preparation of electrolytes. The stock solutions of the Co(II)Tmt-3,4-ppa (concentration of 1×10^{-3} mol dm^{-3}) was prepared by dissolving 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 in distilled water. Phosphoric acid and sodium hydroxide, 0.1 mol dm^{-3} each, were used for pH adjustments. 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

The electrochemical (cyclic voltammetry, chronoamperometry) measurements were carried out with a three-electrode system controlled by the potentiostat/galvanostat Wenking POS 2 (Bank Elektronik, Germany) with CPC-DA software (Bank Elektronik, Germany) in the cell developed and constructed our group for characterization of phthalocyanine as described elsewhere [12]. Saturated calomel electrode (SCE) was used as a reference electrode, platinum

wire as an auxiliary, and basal plane of highly oriented pyrolytic graphite (HOPG) or annealed gold Au(111) as working electrode/support for pyridinoporophyraine compounds. The HOPG electrode was cleaned using adhesive tape Scotch by removing several layers of the surface. The gold substrate Au(111) coated glass slides (Gold arrandeeTM/Au(111) Germany) with a thickness of gold film 250±50 nm in the size of the glass slide 12×12×±0.2 mm 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. All electrochemical measurements were carried out in 0.1 mol dm^{-3} phosphate buffer and deoxygenated by argon at room temperature.

The spectroelectrochemical measurements were performed using the same cell with optical fibre (Ocean Optics, USA) and a fibre optic spectrometer SD 1000 (Ocean Optics, New Zealand). The halogen lamp Fiber Lite PL 800 (Dolan Jenner, USA) was used as the source of irradiation in the range of 400 to 800 nm. The spectra were measured in the backscattering 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 nanomorphology of electrode surfaces was examined by atomic force microscopy (AFM) Multimode Nanoscope IIIa (Bruker, USA) in utilizing tapping mode with silicon tip (OTESPA, 42 Nm-1, 300 kHz, Bruker, USA). The AFM topography was analysed by commercial Nanoscope III Software version5.12r5 (Bruker, USA) and the determination of the surface parameters was performed by using Gwyddion software version 2.41 (Czech Metrology Institute, Brno, Czech Republic).

2.3 Preparation of modified electrode

Pyridinoporphyrazine modified electrodes were prepared by adsorption of mediator on the electrode by drop casting of 1×10^{-3} mol dm^{-3} or 1×10^{-4} mol dm^{-3} CoTmtppa for 30 min or 60 min. Excess solution was removed from the surface. The modified substrate was washed with deionized water, dried at room temperature, and prepared to use. After completion of deposition procedure, the modified electrodes (labelled CoTmtppa/HOPG and CoTmtppa /Au) were further characterized in the buffer solution in the absence of CoTmtppa.

3. Results and discussion

The immobilization of active redox mediator CoTmtppa was carried out spontaneous adsorption on by placing a droplet from aqueous solution on two substrates with different hydrophobicity: annealed gold electrode Au111 (hydrophobic) and highly oriented pyrolytic graphite HOPG (hydrophilic) support surfaces. The modified electrodes (CoTmtppa/HOPG and CoTmtppa/Au) were further

characterized in the buffer solution in the absence of CoTmtppa in the solution. The redox properties of the modified electrodes were studied using cyclic voltammetry and were completed by *in situ* spectroelectrochemistry to have better understanding of deposition processes. Nanomorphology of molecular film on both hydrophilic and hydrophobic surfaces was investigated by *ex situ* atomic force microscopy. Profile analysis made it possible to determine surface roughness parameters and the thickness of the deposited assembly. It was found out that while hydrophobicity of surface has no effect on the redox behaviour, affects nanomorphology, roughness parameters, and thickness of deposited layers.

The electrochemistry of the molecular films shows that the active redox mediator has multi-reduction couples with reversible characters and therefore it could be used to possible practical application as the electrocatalyst. Therefore, electrocatalytic activities of the modified electrodes to hydrogen were studied using cyclic voltammetry and chronoamperometry techniques performed in 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. Finally, it was found that the hydrophobicity of substrate affects the electrocatalytic behaviour.

4. Conclusions

The water soluble non-aggregation *N,N´,N´´,N´´´*-tetramethyltetra-3,4-pyridinoporphyrazinocobalt was immobilization on two substrates: hydrophobic highly oriented pyrolytic graphite and hydrophilic annealed gold electrode. The complex was deposited by spontaneous adsorption from aqueous solution on electrode surfaces. After that, molecular films were characterized electrochemical, spectroelectrochemical, and microscopically techniques. The effects of substrate hydrophobicity on electrocatalytic behaviour to hydrogen were studied. The detailed comparison of results suggests further insight in the processes of thin layers during the modification procedure and electrocatalytic behaviour. It was observed that electrochemical behaviour and electrocatalytic activity of complex to hydrogen were significantly affected by hydrophobicity of electrode substrate.

Acknowledgments

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Investigation of polyaniline-coated silica gel as a stationary phase for separations in different modes of capillary liquid chromatography

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Keywords
capillary liquid chromatography
chromatographic modes
polyaniline-coated silica gel
stationary phase

Abstract
Separation potential of a home-made stationary phase based on polyaniline-coated silica gel particles was investigated in different chromatographic modes of capillary liquid chromatography. Three sets of testing solutes comprised L-tryptophan and structural analogues, positional isomers of aminoacetophenone, and caffeine and its demethylated analogues. The stationary phase exhibits the mixed-mode retention mechanism, thus slightly hydrophilic compounds can be retained in RP mode and vice versa. We successfully separated caffeine, theobromine and theophylline in NP, HILIC, and RP modes. All positional isomers of aminoacetophenone were separated for the first time in NP and RP modes.

1. Introduction

Design, preparation, and characterization of novel stationary phases are integral parts of the modern separation science. Grafting of the original sorbent, most often silica gel, with a broad range of functional groups represents the most common technique. However, it may include a rather complicated, multiple-step preparation process with a low yield. Modification of the original sorbent with a polymer coating of a desirable structure offers a promising alternative. This way comprises a rapid, one-step procedure to obtain a required change of properties. The new sorbent maintains the mechanical stability of the original, usually inorganic, substrate and acquires chemical resistance from the polymer.

Polyaniline (PANI) is a readily synthesized polymer occurring in three different states depending on the degree of protonization and the oxidation state as fully protonated leucoemeraldine, partly protonated emeraldine (Fig. 1) or fully deprotonated pernigraniline. During the polymerization, PANI forms a bulk precipitate and also a thin layer on the surface of the material in direct contact

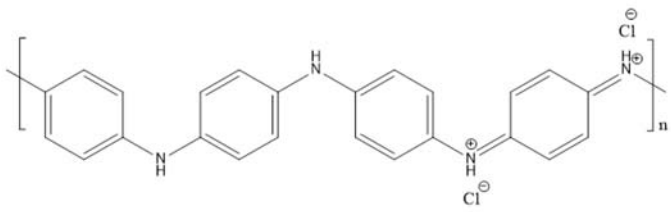

Fig. 1. Chemical structure of partly protonated polyaniline – emeraldine salt.

with the polymerization mixture. Due to this behaviour, PANI can easily be used as a coating material. Emeraldine, a highly stable, semiconductive form of polyaniline, has been investigated as a surface modifier for multiple applications, such as flexible electrodes, anticorrosive coatings or catalysts [1]. Some attempts have been made to employ polyaniline coatings as an inner-wall modifier in CZE [2] and continuous flow analysis [3] or as a sorbent for SPME [4], and TLC [5]. Stejskal et al. [6] developed a simple coating method based on in situ polymerization of PANI on spherical silica gel dispersed in a reaction mixture containing aniline hydrochloride and ammonium persulfate. Sowa et al. [7] adopted the latter approach to synthesize the stationary phase and studied its potential application in non-suppressed ion chromatography.

In our previous work, we characterized polyaniline-coated silica gel (PANI-SiO_2) spherical particles using the linear solvation energy relationship (LSER) approach extended with ionic interactions terms in hydrophilic interaction liquid chromatography (HILIC) mode. A mixed-mode retention mechanism was observed. Individual interactions taking place here correspond to the structure of PANI-SiO_2; specifically benzene rings enable π-π stacking, amino and imino moieties act as hydrogen bond (H-bond) acceptors or cations, and underivatized silanols behave as H-bond donors or anions. Especially anionic and zwitterionic solutes have a considerable retention under HILIC conditions.

In this study, we have investigated the separation potential of the stationary phase using structurally similar compounds also in normal phase (NP) and reversed phase (RP) modes.

2. Experimental

2.1 Reagents and chemicals

Acetonitrile, methanol (both of HPLC gradient grade) and all solutes used in this work (all of analytical grade purity) were purchased from Sigma-Aldrich. Formic acid, hydrochloric acid (35%) and tris(hydroxymethyl)aminomethan were supplied by Lachner (Neratovice, Czech Republic). The deionized water was purified with a Milli-Q water purification system from Milli-pore. Tris-HCl buffer was prepared by dissolving the appropriate amount of Tris in deionized water; the

required pH value was adjusted by titration with HCl. Solute solutions were prepared in a concentration range of 0.05–1.00 mg/mL either in pure acetonitrile (ACN) or in ACN/water mixture (50/50; *v/v*), depending on their solubility.

2.2 Instrumentation

The preparation of PANI-SiO_2 sorbent and the capillary packing procedure are briefly described below. Original bare silica gel (Hypersil, spherical, average particle size 5 μm, average pore size 12 nm) was modified with PANI coating by in situ chemical polymerization of aniline hydrochloride; ammonium persulfate was employed as the polymerization initiator. The sorbent was then collected on a filter and repeatedly rinsed with small portions of diluted hydrochloric acid, organic solvents and water to remove all possible impurities. Prepared PANI-SiO_2 sorbent was then slurry packed into a column made of a polyimide-coated fused silica capillary (320 μm I.D.) with an outlet quartz wool frit inserted into a PEEK union. As the slurry solvent 50% methanol (*v/v*) was used to obtain a slurry concentration of 0.02 g/mL. The column was packed at a pressure of 25 MPa using 65% acetonitrile (*v/v*) as a packing solvent.

Chromatographic measurements were performed using an Agilent 1200 HPLC System consisting of a degasser, quaternary pump, automated injector, column oven and diode array detector. The 3DHPLC ChemStation Software (Agilent Technologies) was used for acquisition and analysis of the experimental data. The injection volume was 0.1 μL for all the solutes and the flow rate varied in the range of 5–15 μL/min. The capillary column was thermostated at 25 °C or 50 °C. UV detection was performed at 200 nm, 230 nm, and 265 nm. The dead time was determined using a system peak.

3. Results and discussion

L-tryptophan (Trp) and its three derivatives differing in functional group bound to amino or carboxyl moiety of Trp, i.e. L-tryptophan methyl ester hydrochloride (MeE-Trp), *N*-acetyl-L-tryptophan (NAc-Trp) and *N*-α-(*tert*-butoxycarbonyl)-L--tryptophan (Boc-Trp), were chosen to assess the retention ability of PANI-SiO_2 as the stationary phase. Chemical structures of relevant solutes are depicted in Fig. 2. These compounds have diverse values of pK_a and distribution coefficient (log $D_{o/w}$), see Table 1. Therefore, combination in testing can provide a deeper insight into retention properties of the stationary phase.

As PANI-SiO_2 offers the mixed-mode retention mechanism, we assumed that the retention factor curve should pass minimum when the ratio of organic/aqueous constituent in the binary eluent is close to unity. The assumption was indeed confirmed (Fig. 3). The retention of Boc-Trp and NAc-Trp is considerable as they both are hydrophobic in an acidic RP mobile phase with high content of water. In addition, the presence of *tert*-butyl group in Boc-Trp may contribute to the

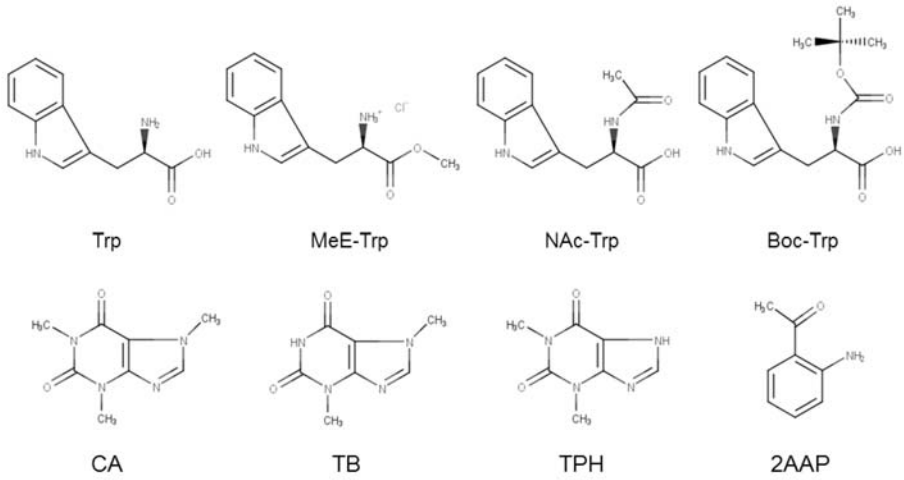

Fig. 2. Chemical structures of used solutes .

Table 1
Values of pK_a of functional moieties and distribution coefficients of solutes used for retention factor measurement under different mobile phase composition.

Solute	pK_a		log $D_{o/w}$	
	–COOH	$-NH_2$	pH = 2.5	pH = 8.0
Trp	2.54	9.40	–1.79	–1.58
MeE-Trp		6.92	–2.12	1.25
NAc-Trp	4.12		1.07	–2.39
Boc-Trp	4.13		2.53	–0.93

increased retention. In contrast to this, Trp and MeE-Trp are retained poorly as they are hydrophilic under given conditions. Moreover, protonated nitrogen atoms of PANI repel underivatized, positively charged amino moieties of Trp and MeE-Trp. On the other hand, all solutes are retained to a certain extent in HILIC mode. The retention is lowest for Boc-Trp as it has only partly dissociated carboxylic group and its carbonyl oxygen, able to act as an H-bond acceptor, is shielded by a branched-alkyl residue. MeE-Trp's interaction with stationary phase is limited by its methyl esterification. Although NAc-Trp has pK_a(–COOH) value equal to Boc-Trp, its acetyl oxygen can act as a strong H-bond acceptor. Native Trp has pK_a(–COOH) value close to pH value of the mobile phase buffer so we can suppose its significant involvement in ionic or H-bond interactions.

Boc-Trp and NAc-Trp are hydrophilic in a basic eluent which is given by dissociation of carboxylic groups, on the contrary, MeE-Trp is hydrophobic. It seems that ionic interactions have a prevailing effect over hydrophobicity as can be seen on retention of solutes in RP mode. The higher retention may also be given

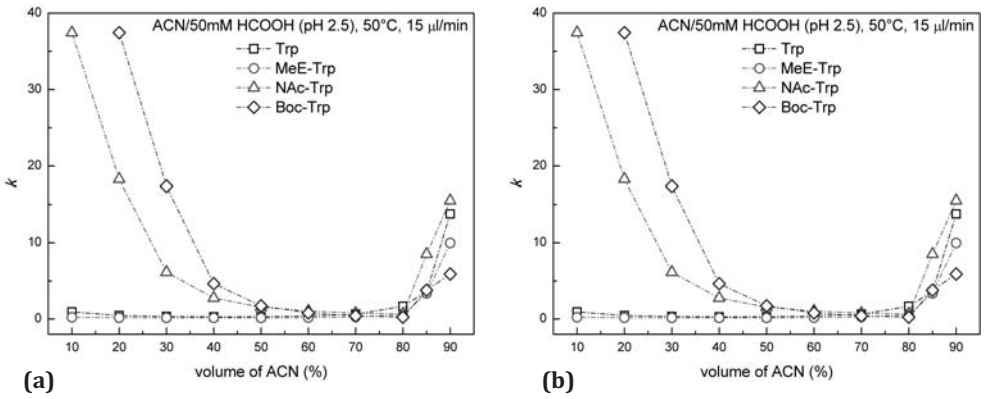

Fig. 3. Dependence of retention factor of Trp and its derivatives on content of ACN in the eluent with (a) acidic or (b) basic aqueous constituent.

by substantially lower protonation of amino moieties of solutes (except for Trp) hereby leading to their suppressed repulsion with positively charged groups of stationary phase. Ionic interactions are dominant in HILIC mode, so MeE-Trp has lowest retention. The values of distribution coefficient and possible H-bonding support this presumption.

In the second part of the study, two sets of structurally similar neutral compounds (Fig. 2) having slightly hydrophilic or hydrophobic character were chosen for investigation of the separation potential of PANI-SiO_2 in NP, HILIC and RP modes. The first set consisted of caffeine (CA, log $D_{o/w}$ = –0.79), theobromine (TB, log $D_{o/w}$ = –1.03), and theophylline (TPH, log $D_{o/w}$ = –1.03), and the second one of 2´-aminoacetophenone (2AAP, log $D_{o/w}$ = 1.22), 3´-aminoacetophenone (3AAP, log $D_{o/w}$ = 0.57), and 2´-aminoacetophenone (4AAP, log $D_{o/w}$ = 0.57). The resolution higher than 1.5 was achieved for both sets of solutes in NP mode using pure ACN (mild elution strength) as the eluent (Fig. 4). CA and its derivatives are more retained and separated than 2AAP and its positional analogues since they are more polar. In case that solutes have similar log $D_{o/w}$ value, other effects on retention have to be considered, e.g. dislocation of electron density given by mesomeric effect for AAPs or accessibility of NH moiety (7-labeled nitrogen in TPH structure) for H-bonding.

In HILIC mode, the resolution of CA, TB and TPH was somewhat lower, but the overall retention and sensitivity was higher. Poor retention and separation of AAPs may be related to their low polarity.

The expected shift of the elution order and a substantial increase of the retention were observed for AAPs in RP mode. It is worth noting that 2AAP and 4AAP, having the same mesomeric effect, were baseline resolved.

Despite the significant change of the eluent composition, TPH remained the most retained solute of the set even in RP mode. We may find explanation in the very strong H-bond interactions between TPH and the mixed-mode stationary phase.

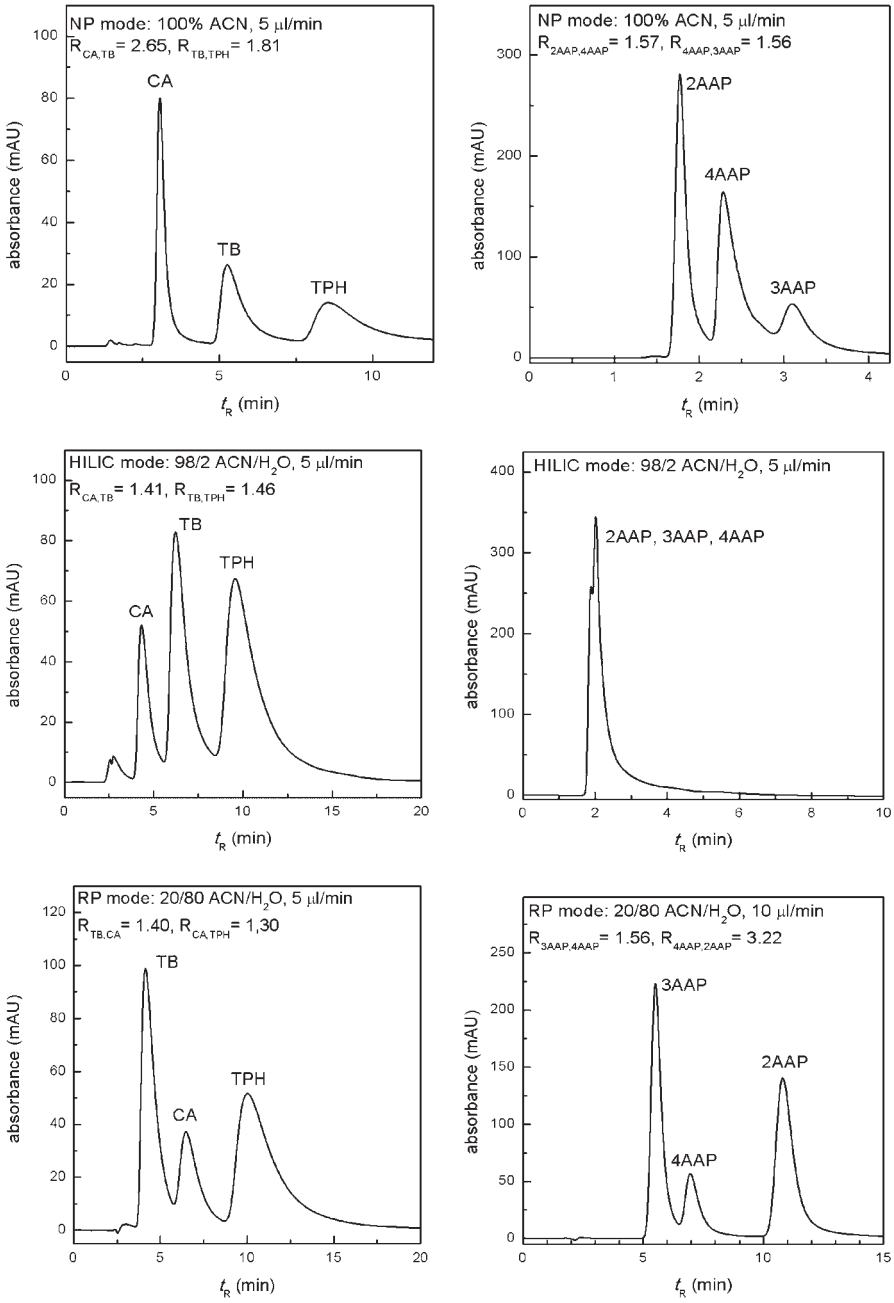

Fig. 4. Different chromatographic modes used for separation of CA and its demethylated derivatives (left column) and positional isomers of AAP (right column). Separation conditions: PANI-SiO_2 17 cm × 320 μm I.D., average particle size 5 μm (PANI layer thickness ~ 180 nm); temperature 25 °C, UV detection at 265 nm for CA, TB, TPH and 230 nm for AAPS.

4. Conclusions

As PANI-SiO_2 stationary phase exhibits the mixed-mode retention mechanism it is not possible to simply predict the retention of solutes based only on their polarity. Other factors including ionic interactions, H-bonding, and structural isomerism have to be taken into account as well. Using this stationary phase, we investigated its separation potential on structurally similar solutes having different polarity. We found out that even hydrophilic solutes can be remarkably retained in RP mode. Next, we successfully separated caffeine, theobromine and theophylline in NP, HILIC, and RP modes. All three positional isomers of aminoacetophenone were separated for the first time in NP and RP modes.

Acknowledgments

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Study of the interaction between DNA and 4-nitrobiphenyl using voltammetry at a hanging mercury drop electrode

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Keywords
DNA
hanging mercury drop electrode
interaction
4-nitrobiphenyl
voltammetry

Abstract
This paper presents a set of methods for investigation of the interaction between double-stranded DNA and 4-nitrobiphenyl and of DNA damage caused by this interaction. For this purpose, differential pulse, cyclic, and alternating current voltammetry, and chronocoulometry at a hanging mercury drop electrode (HMDE) or DNA modified HMDE were employed. The interaction of DNA with reduction intermediates of 4-nitrobiphenyl was studied, too. The mechanism of the electrochemical reduction of 4-nitrobiphenyl was investigated using cyclic voltammetry. Moreover, the values of the standard rate constant of the reduction of 4-nitrobiphenyl and the diffusion coefficient were calculated.

1. Introduction

4-Nitrobiphenyl belongs to the group of nitrated aromatic hydrocarbons. Nitrated aromatic hydrocarbons are mutagenic [1, 2], as they are enzymatically transformed to free anion-radicals inducing genotoxic effects [3]. By the reduction of the aromatic nitro group, species reacting with cellular macromolecules are formed [3]. Therefore, the importance of monitoring and of investigation of the biological effects of nitrated aromatic hydrocarbons is high [4]. Electrochemical reduction and determination of 4-nitrobiphenyl have been studied previously [5, 6]. 4-Nitrobiphenyl is a biologically active, genotoxic, and ecotoxic compound. It is not carcinogenic to human [7], however, its carcinogenic effects on animals were observed [8].

Damage of double-stranded DNA (*ds*DNA) can be caused by physical or chemical agents [9], and may result in its degradation (fragmentation) or denaturation (unwinding) [10]. For investigation of interactions of DNA with organic compounds and for DNA damage detection, electrochemical techniques [11–13]

are very useful; the most frequently used are cyclic (CV) [12], differential pulse (DPV) [14], and alternating current voltammetry (ACV) [15].

The interaction can be also studied by determining kinetic and thermodynamic parameters, i.e., standard rate constant ($k°$) and diffusion coefficient (D). The influence of the presence of DNA in the solution of the analyte on the values of these quantities is investigated. From the value of $k°$, it is possible to estimate the electrochemical reversibility of the reaction [16].

The main aim of this study was the development of voltammetric methods for the investigation of the interaction between DNA and 4-nitrobiphenyl and its study, and for the detection of consecutive DNA damage.

2. Experimental

2.1 Reagents and chemicals

Methanolic stock solution (c = 1 mmol L^{-1}) of 4-nitrobiphenyl (99%, Merck) was prepared by dissolving the pure substance in methanol (99.9%, Merck). The stock solution of DNA (stored at 4 °C, γ_{DNA} = 10.0 mg mL^{-1} corresponds to the concentration of DNA base pairs 0.04 mol L^{-1}) was prepared by dissolving low molecular weight salmon sperm *ds*DNA (Product Number 31149, Sigma-Aldrich; stored in a fridge at 4 °C) in 0.3 mol L^{-1} NaCl (Lach-Ner, Czech Republic). Phosphate buffer (PB, 0.01 mol L^{-1}, pH = 7.0) was prepared from $NaH_2PO_4.H_2O$ (Sigma-Aldrich) and $Na_2HPO_4.10H_2O$ (Lach-Ner, Czech Republic). Dimethyl sulfoxide (Lachema, Czech Republic) and NaOH (Penta, Czech Republic) were used. All used chemicals were of p.a. purity. Deionized water produced by a Milli-Q Plus system (Millipore, USA) was used in all cases. If not stated otherwise, all solutions were stored in glass bottles in the dark at laboratory temperature.

2.2 Instrumentation

All measurements were carried out with an Autolab PGSTAT10 potentiostat/galvanostat connected to a Metrohm 663 VA Stand (both Metrohm Autolab) in a three-electrode system with a Ag|AgCl (3 mol L^{-1} KCl) reference electrode (Elektrochemické Detektory, Czech Republic), a platinum wire auxiliary electrode (Monokrystaly, Czech Republic), and a hanging mercury drop electrode (HMDE) as a working electrode (Metrohm Autolab). The mercury drop surface area was 0.49 mm^2. If not stated otherwise, the scan (v) rate was 20 mV s^{-1}. The modulation amplitude 50 mV and the modulation time 80 ms were used for DPV. The modulation amplitude 50 mV, the frequency 230 Hz, and harmonic 1 were used for ACV. The potentiostat was computer-controlled by the NOVA 1.11 (measurements of DPV, CV, and chronocoulometry) and NOVA 2.0 (measurements of CV and ACV) software (Metrohm Autolab). All potentials were referred to the reference Ag|AgCl electrode mentioned above.

2.3 General procedures

Measurements at HMDE: The measured solution was prepared into a 10 mL volumetric flask, and transferred into a voltammetric cell. Then, an appropriate volume of DNA stock (10.0 mg mL^{-1}) solution was added. Oxygen was removed by bubbling with nitrogen (purity class 4.0, Linde Gas, Czech Republic) for 5 min before each measurement, and a nitrogen atmosphere was then maintained above the solution in the cell.

Measurement at DNA modified HMDE (DNA-HMDE): The HMDE drop was created in a drop of the DNA solution (1 mg mL^{-1}), immediately rinsed with deionized water, and transferred into a base electrolyte. Before some measurements, DNA-HMDE was incubated in the analyte solution. All measurements were performed after 2 min bubbling with nitrogen.

All measurements were performed three times. The values for the construction of functional dependences were calculated as arithmetic averages. All measurements were carried out at laboratory conditions. The statistical quantities were calculated at the significance level $\alpha = 0.05$.

3. Results and discussion

3.1 Differential pulse and cyclic voltammetry of 4-nitrobiphenyl at a hanging mercury drop electrode in the absence and presence of DNA in the solution

The optimum medium was found, via studying changes of the peak current (I_p) and the peak potential (E_p) of 4-nitrobiphenyl using DPV, as 0.01 mol L^{-1} PB (pH = 7.0):methanol (8:2). The interaction time (t_{int}) 5 min, used for removing oxygen before each measurement, was sufficient for the interaction of 4-nitrobiphenyl with DNA; I_p was not affected by a temperature (tested in range from 16 to 40 °C) and by the concentration of 4-nitrobiphenyl.

Using DPV, it was found that the presence of DNA in the solution of 4-nitrobiphenyl (10 μmol L^{-1}) resulted *(i)* in a decrease of I_p of 4-nitrobiphenyl; *(ii)* in a peak of cytosine and adenine moieties (peak CA) formation at ca −1500 mV; and *(iii)* in a significant shift of E_p to less negative values at $\gamma_{DNA} > 100$ μg mL^{-1}. The interaction of DNA with 4-nitrobiphenyl results in the DNA structure changes, but a DNA–4-nitrobiphenyl complex is not formed.

Cyclic voltammograms of 4-nitrobiphenyl (10 μmol L^{-1}) were measured in the absence and presence of DNA (5 and 100 μg mL^{-1}) in PB:methanol (8:2). I_p of 4-nitrobiphenyl and the peak CA current (I_{CA}) were studied in t_{int} from 0 to 30 min in the presence of DNA (5 μg mL^{-1}). The change of I_p of 4-nitrobiphenyl was not significant, and I_{CA} decreased to ca 75% (after 5 min). After 30 min, the decrease was the same (summarized in Table 1). The decrease of I_{CA} can be caused by the DNA aggregation with 4-nitrobiphenyl, leading to the DNA structure changes. The resulting DNA behavior can be affected by the presence of 4-nitrobiphenyl, and vice versa.

Table 1
An overview of the effects of the used DNA damaging agents on the relative changes of the peak CA and peak 3 currents. Experimental conditions are described in corresponding Sections. All values are related to the blank measurements.

DNA damaging agent	Relative change of the peak current [%]		Section
	Peak CA	Peak 3	
4-Nitrobiphenyl (DNA in solution)	75±6	–[a]	3.1
Temperature	84±1	81±8	3.3
4-Nitrobiphenyl	–[b]	–[a]	3.3
CV from −300 to −800 mV	–[b]	117±1	3.3
CV from 0 to −800 mV	64±14	89±2	3.3
Electrolysis at −800 mV	228±13	84±2	3.3

–[a] Not investigated.
–[b] The change of the peak current was not significant.

The reduction of 4-nitrobiphenyl in the absence and presence of DNA is irreversible. Trends in the E_p shift and the change of I_p of 4-nitrobiphenyl were the same as the ones observed using DPV. In comparison with DPV, CV gave better evaluable peak CA. Therefore, for the measurements evaluating peak CA, CV was used. CV of 4-nitrobiphenyl in the absence and presence of DNA was recorded with scan rate from 5 to 1000 mV s^{-1}. From the slopes of plotting log ($-I_p$) of 4-nitrobiphenyl on log v (summarized in Table 2), it can be concluded that in both the absence and presence of DNA, the electrochemical reduction is controlled by both adsorption and diffusion [16].

3.2 Determination of kinetic parameters of the reduction of 4-nitrobiphenyl in the absence and presence of DNA in the solution

Solutions of 4-nitrobiphenyl (10 μmol L^{-1}) with DNA (0, 5, and 100 μg mL^{-1}) were studied. The values of D of 4-nitrobiphenyl were calculated from the Anson's

Table 2
Kinetic parameters of the reduction of 4-nitrobiphenyl (10 μmol L^{-1}) in the absence and presence of DNA. Slopes (including standard errors) of the dependences of the log ($-I_p$) of 4-nitrobiphenyl on the log v were evaluated in the range of v from 20 to 100 mV s^{-1}. Measured in PB:methanol (8:2) using CV at HMDE.

Analyte	Slope of log ($-I_p$) vs. log v	$D\times10^5$ [cm^2s^{-1}]	$k°\times10^5$ [cm s^{-1}]
4-Nitrobiphenyl	0.68±0.03	2.3±0.1	1.5±0.1
4-Nitrobiphenyl with DNA (5 μg mL^{-1})	0.68±0.02	1.9±0.1	1.0±0.1
4-Nitrobiphenyl with DNA (100 μg mL^{-1})	0.67±0.01	2.0±0.1	1.3±0.1

equation [17] (using data from chronocoulometry, with reaction times from 4 to 64 s). Values of $k°$ were determined using data from CV [18]. The values of D and $k°$ are summarized in Table 2. It is concluded that the reaction is electrochemically quasi-reversible, and it is not affected by the presence of DNA in the solution [16]. The DNA–analyte complex is not formed, and the competitive sorption of 4-nitrobiphenyl and DNA on the electrode surface occurs.

3.3 Study of the interaction of DNA with 4-nitrobiphenyl or its reduction intermediates at a DNA modified hanging mercury drop electrode

CV in PB and ACV in 0.3 mol L^{-1} NaCl with 0.05 mol L^{-1} NaH_2PO_4 (adjusted to pH = 8.5 by titration with 0.2 mol L^{-1} NaOH) were employed, investigating I_{CA} and the peak 3 current (I_3), respectively. Optimal v = 200 mV s^{-1} was chosen for CV, optimal measurement parameters of ACV were taken from [19], with v = 20 mV s^{-1}.

Firstly, the influence of DNA denaturation processes of DNA (by the increased temperature) on I_{CA} and I_3 was studied (see Table 1). Typically, I_{CA} and I_3 are increasing. In comparison to previously published works investigating DNA damage, our voltammograms are similar to voltammograms of single-stranded DNA [20] (probably because of the shortness of the used salmon sperm *ds*DNA). ACV is a good tool for the detection of single-/double-stranded DNA form, but in this case, CV is more sensitive and reliable for the study of DNA damage.

Using CV, interaction of DNA with 4-nitrobiphenyl was studied at DNA-HMDE in PB. The relative change of I_{CA} was not significant, and was affected neither by the concentration of 4-nitrobiphenyl nor by the incubation time.

Using CV (20 cycles from −300 mV to −800 mV or from 0 to −800 mV, with v = 50 mV s^{-1}) or potentiostatic electrolysis (for 10 min at −800 mV) during the incubation of DNA-HMDE in the solution of 4-nitrobiphenyl (10 μmol L^{-1}) in PB, the reduction intermediates of 4-nitrobiphenyl were generated. After that, changes of I_{CA} and I_3 were investigated. The interaction of DNA with the reduction intermediates was conclusively confirmed. All results are summarized in Table 1.

4. Conclusions

Using CV, DPV, and ACV, the interaction of DNA with 4-nitrobiphenyl was studied in PB or PB:methanol (8:2) at HMDE and at DNA-HMDE. The interaction results in I_p of 4-nitrobiphenyl and I_{CA} decreases. Electrochemical reduction of 4-nitrobiphenyl is a quasi-reversible process controlled by both adsorption and diffusion in the absence of DNA as well as in the presence of DNA. Using CV in PB and ACV in 0.3 mol L^{-1} NaCl with 0.05 mol L^{-1} NaH_2PO_4 (pH = 8.5), I_{CA} and I_3 were investigated at DNA-HMDE. After incubation in 4-nitrobiphenyl solution, no significant changes of I_{CA} and I_3 were observed. The interaction of DNA with reduction intermediates of 4-nitrobiphenyl was confirmed. It can be concluded that the interaction of DNA with 4-nitrobiphenyl results in formation of a DNA aggregate.

Acknowledgments

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Voltammetric determination of cancer biomarker 5-hydroxyindole-3-acetic acid at screen-printed carbon electrodes

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Keywords
differential pulse voltammetry
5-hydroxyindole-3-acetic acid
screen-printed carbon electrodes
tumor biomarker

Abstract
Tumor biomarker 5-hydroxyindole-3-acetic acid (5-HIAA) is a breakdown product of serotonin; level of this neurotransmitter can predict carcinoid tumors. 5-HIAA has been determined at screen-printed carbon electrodes in the optimum medium found: Britton-Robinson buffer (0.04 mol l^{-1}, pH = 3.0). Dependence of the peak current on the concentration of 5-HIAA was linear in the concentration region from 0.1 to 100 μmol l^{-1}, with the limit of quantification of 0.2 μmol l^{-1}.

1. Introduction

5-Hydroxyindole-3-acetic acid (5-HIAA) is the product of oxidative deamination of serotonin (5-hydroxytryptamine) and is excreted in urine [1]. Phenolic structure suggests its oxidizability at carbon electrodes, which can be used for its determination [2]. Determination of urinary catecholamine and serotonin metabolites can be useful tool in the clinical practice for prediction of neuroblastic and carcinoid tumors [3]. Serotonin is metabolite of tryptophan implicated in important physiological processes (e.g. homeostasis and regulation of the intestinal motility). It is a neurotransmitter in a central nervous system, where it plays a role in behavioral control, sleep, and feeding. Except prediction of tumors, determination of serotonin and 5-HIAA in biological fluids is effective for the diagnosis of hypertension, depression, migraine, and Tourette syndrome [1]. The normal concentration range for urinary 5-HIAA is from 17.8 to 58.3 μmol l^{-1} according to paper [4]. The normal concentrations of catecholamine metabolites (vanillylmandelic acid, homovanillic acid, and 5-HIAA) are between 1.3 and 7.6 mg l^{-1} (6.7 and 39.8 μmol l^{-1}) according to paper [5]. Measurement of urinary 5-HIAA excretion is a biochemical marker of the carcinoid syndrome, but excretion is

influenced by the dietary intake of serotonin-rich food (banana, pineapple, tomato, kiwi fruit, and walnut) and by the pharmacological actions of many drugs which increase (diazepam, aminophenols, naproxen, etc.) or decrease (salicylates, chlorpromazine, L-DOPA, etc.) the level of 5-HIAA. To avoid difficulties in the interpretation of 5-HIAA results, this food should be excluded from the diet and drug therapy interrupted before taking a urine sample for determination of 5-HIAA [6, 7]. The most common methods for determination of 5-HIAA in clinical laboratories are HPLC-ED and GC-MS [8].

Voltammetric determination of 5-HIAA was performed at commercially available screen-printed carbon electrodes (SPCE, type DRP 110, DropSens) which are suitable tools for determination of various organic compounds in biological fluids [9]. SPCE can be used outside a centralized laboratory; they are small and easy transferable, exhibit quick response, and high sensitivity. If necessary, they can be chemically and/or biologically modified. They are disposable, so there is no problem with their passivation [10].

2. Experimental

2.1 Reagents and chemicals

The stock solution (1 mmol l^{-1}) of 5-HIAA was prepared by dissolving 4.78 mg of 5-HIAA (CAS Number: 54-16-0; Product Number: H8876, ≥ 98%, Sigma-Aldrich) in 25 ml of deionized water. All measurements were performed in Britton-Robinson buffer (BRB) prepared in a usual way (i.e., by mixing a solution of 0.04 mol l^{-1} phosphoric acid, 0.04 mol l^{-1} acetic acid, and 0.04 mol l^{-1} boric acid with the appropriate amount of 0.2 mol l^{-1} sodium hydroxide solution). To prepare measured solutions of 5-HIAA, appropriate amount of its stock solution was placed into a volumetric flask and filled up to the mark with BRB of the required pH.

2.2 Instrumentation

The voltammetric measurements were performed using a computer controlled Eco-Tribo Polarograph with Polar Pro software, version 5.1. Differential pulse voltammetry (DPV) was performed using SPCE (type DRP 110, DropSens) with a three-electrode system comprising of a carbon working electrode (4 mm diameter), a carbon counter electrode, and a silver reference electrode. Prepared solutions of 5-HIAA were transferred into a voltammetric vessel, and the voltammograms were recorded using DPV from −200 to 1600 mV, with pulse height +50 mV, scan rate 20 mV s^{-1}, pulse width 100 ms, and current range 100 μA. For calculating calibration curve parameters and graphic expressions of results, Microsoft Office Excel 2010 (Microsoft Corporation) and OriginPro 8.0 (OriginLab Corporation, USA) were used. The limit of quantification (*LOQ*) was calculated as $LOQ = 10s/a$, where s is the standard deviation of 10 repetitive measurements of

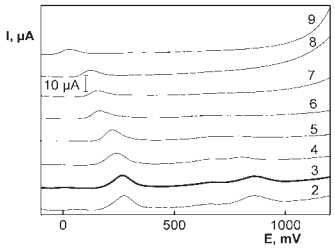

Fig. 1. DP voltammograms of 5-hydroxyindole-3-acetic acid (0.1 mmol l^{-1}) measured at screen-printed carbon electrode in Britton-Robinson buffer at different pH (pH is given above the curves).

the lowest measurable concentration, and *a* is the slope of the calibration curve [11].

3. Results and discussion

BRB was chosen as a base electrolyte. At first, DP voltammograms of 5-HIAA in BRB at different pH were measured. The pH dependence was measured from pH = 2.0 to pH = 9.0; at pH > 9, the peak of 5-HIAA was not evaluable (Fig. 1). The optimum medium for the determination of 5-HIAA was found to be BRB at pH = 3.0. In acidic pH, there are two well-separated peaks of 5-HIAA.

Thereafter, the dependence of the peak current on the analyte concentration was measured in BRB at pH = 3.0 in the concentration range from 0.1 to 100 μmol l^{-1} (Fig. 2). The dependence was linear in this concentration range. DP voltammograms of the 5-HIAA in the concentration range from 10 to 100 μmol l^{-1} are shown in Fig. 2A. The first peak is significant, well developed, and useful for analytical purposes. Fig. 2B and 2C depict the concentration ranges from 1 to 10 μmol l^{-1} and from 0.1 to 1 μmol l^{-1}, respectively. Three peaks of 5-HIAA were observed, however, only the peak at a potential around 270 mV was evaluated for the calibration dependence construction and the *LOQ* calculation because evaluation of the other peaks is more difficult and leads to less precise results. The

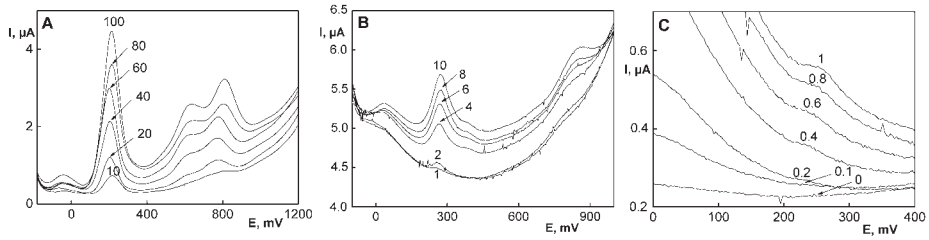

Fig. 2. DP voltammograms of 5-hydroxyindole-3-acetic acid measured at screen-printed carbon electrode in Britton-Robinson buffer at pH = 3.0 in the concentration ranges from (A) 10 to 100 μmol l^{-1}, (B) 1 to 10 μmol l^{-1}, and (C) 0.1 to 1 μmol l^{-1}; the numbers next to the curves correspond to the analyte concentrations in μmol l^{-1}.

obtained figures of merit for the determination of 5-HIAA (1st peak) at SPCE in BRB at pH = 3.0 in the concentration range from 0.1 to 100 μmol l^{-1} are: slope of 35.8 mA l mol^{-1}, intercept of 116.0 nA, and *LOQ* of 0.2 μmol l^{-1}.

4. Conclusions

Voltammetric determination of tumor biomarker 5-hydroxyindole-3-acetic acid (5-HIAA) represents a useful approach for prediction of diseases and could help to start their treatment in time. This work describes the determination of 5-HIAA using DPV at SPCE. The optimum medium was found to be BRB at pH = 3.0. The LOQ, obtained by evaluation of the first peak of 5-HIAA, was 0.2 μmol l^{-1}. The calibration dependence was linear in the tested concentration range (from 0.1 to 100 μmol l^{-1}). The obtained results prove that DPV at SPCE is sensitive enough for monitoring of 5-HIAA and suggest possible voltammetric monitoring of this tumor marker in various biological matrices. The application of the newly developed methods for urine samples would require some preliminary separation and preconcentration, solid phase extraction and/or various forms of membrane separation being the most suitable candidates for this purpose. Obtaining samples of urine is noninvasive and simple. DPV at SPCE can thus be also used for in situ measurements.

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Voltammetric studies of oxidation of *p*-cresol at boron doped diamond electrode

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Keywords
boron concentration
boron doped diamond
p-cresol
voltammetry

Abstract
Cresols are toxic environmental pollutants. In this contribution possibilities of quantitation of *p*-cresol at BDD electrodes with different boron concentration are presented. Well shaped anodic voltammetric peaks were obtained for both, anodically pre-treated semi-conductive 500 ppm and 1000 ppm, and metallic-type 4000 ppm and 8000 ppm BDD electrodes in acidic media. Limits of detection for DP and DC voltammetry are lower than 0.1 μmol l^{-1} for the preferred boron doped diamond film deposited at B/C ratio 4000 ppm.

1. Introduction

Cresols are organic aromatic compounds classified by the US-EPA as persistent and toxic chemicals, showing chronic effects at 12 mg l^{-1} (ref. [1, 2]). There are three types of methylphenol isomers exhibiting close chemical and physical properties. The mix of these isomers is commonly used for the wood impregnation and for production of polymers, disinfections and herbicides. Wastewater from these industries and from coal conversion has a high concentration of cresols [1].

Boron doped diamond (BDD) electrodes possess a great combination of properties for electrochemical analysis, e.g. large potential window, especially in the anodic part, low proclivity to the fouling of the electrode surface and to oxygen reduction, low background current, and easily electrochemically renewable electrode surface in comparison with other carbon based electrodes [3]. The products of oxidation of phenolic compounds are responsible for electrode fouling. Their oxidation is a complicated process with many steps involving formation of phenoxy-type radicals and their electrochemical behaviour depends on many factors such as pH of the solution, type of the electrode, and current density [4]. For BDD electrodes boron concentration determining semi-conductive or metallic type of conductivity and surface morphology, and further type of activation of the surface are the crucial factors influencing their electrochemical properties [4–6].

In this contribution, voltammetric methods for the determination of *p*-cresol are optimized and BDD electrodes with both types of conductivity compared in terms of peak parameters and sensitivity.

2. Experimental

2.1 Reagents and chemicals

Standard solution of *p*-cresol (Sigma Aldrich, ≤95%; c = 1×10^{-4} mol l^{-1}) was prepared in deionised water. As a supporting electrolyte the Britton-Robinson (BR) buffers were used (concentration 0.04 mol l^{-1} of each acidic component mixed with 0.2 mol l^{-1} solution of sodium hydroxide to the required pH, all chemicals p. a., Lach-Ner, Czech Republic).

2.2 Instrumentation

For this study four boron doped diamond electrodes prepared using microwave plasma assisted chemical vapour deposition procedure (MPCVD) at B/C ratio: 500 ppm, 1000 ppm, 4000 ppm, 8000 ppm (AS CR, Institute of Physics, Czech Republic) were used. The latter electrode was used for optimization experiments. All electrochemical measurement was performed in a three-electrode arrangement using the potentiostat Autolab with the software Nova 1.11 and 2.0 (Metrohm Autolab): BDD as working electrode (geometric area 5.72 mm^2), argentchloride electrode (3.0 mol l^{-1} KCl) as reference and platinum wire as auxiliary electrode (both Elektrochemické detektory, Turnov, Czech Republic) and three electrochemical methods were used for this study: differential pulse voltammetry with the scan rate of 20 mV s^{-1} and modulation amplitude of 25 mV, DC voltammetry at 50 mV s^{-1}, square wave voltammetry (SWV) with modulation amplitude 0.07 V and frequency 25 Hz. The BDD surface was in concordance with other cresols [7] anodically pre-treated applying +2.4 V at working electrode for 5 min in 0.5 mol l^{-1} sulfuric acid at the beginning of each working day and activated by 1 min in-situ pre-treatment at the same potential before each individual measurement.

3. Results and discussion

3.1 pH dependence

The pK_a value of *p*-cresol is 10.26 [8] and thus the pH of the measured solution influences its dissociation and consequently the electrochemical behaviour. pH dependence of oxidation potential of *p*-cresol in the pH range of 2.0 to 12.0 is depicted in Fig. 1. A relatively high oxidation potential of ca +1.1 V for the most acidic solution has been observed with consequent shift to lower potentials with

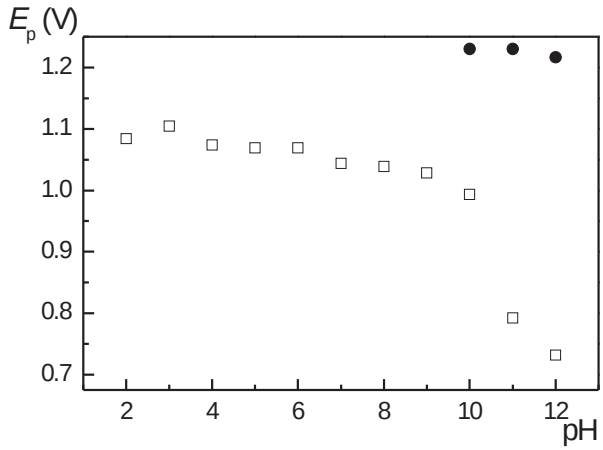

Fig. 1. Peak potential dependence on pH of solution. Potentials from DP voltammetry of *p*-cresol ($c = 1\times10^{-4}$ mol l^{-1}) in Britton-Robinson buffer of different pH at BDD electrode deposited at B/C ratio 8000 ppm. Potential of the first main (□) and second (●) oxidation peak.

increasing basicity of the supporting electrolyte up to the pH 10.0. At higher pH values second peak appears and simultaneously a drop of oxidation potential of the main peak of about 250 mV is observed as consequence of the ionization of the molecule and its attraction to the positively charged electrode. Acidic medium of pH 2.0 was chosen as optimal and other measurements were made in acidic part of the BR buffer.

3.2 Influence of boron concentration

The boron concentration in diamond film influences significantly the electron transfers kinetics of phenolic compounds [9, 10] and thus has a large influence on the peak potential and peak current. Fig. 2 depicts DP voltammograms of *p*-cresol measured at semi-conductive 500 ppm and 1000 ppm, and metallic-type 4000 ppm, and 8000 ppm BDD electrodes. The signal of *p*-cresol is well developed at all BDD electrodes, as in the case of other cresols [7]. While the latter electrodes exhibit similar peak currents, a substantial difference arises among them and both semi-conductive films 500 ppm and 1000 ppm. In this case the boron concentration has not significant influence on the peak potential.

3.3 Calibration dependences

Calibration dependences were measured in acidic part of BR buffer with all BDD electrodes (500 ppm–8000 ppm) using all mentioned voltammetric methods in the concentration range 2.5 μmol l^{-1} to 200 μmol l^{-1}. Achieved limits of detection are given in the Table 1. The values suggest that the electrodes with metallic type

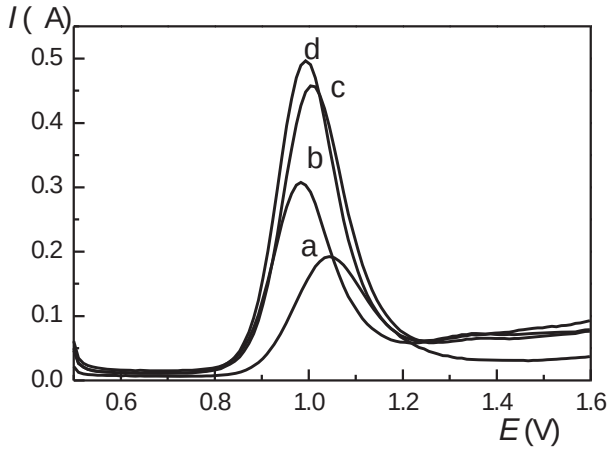

Fig. 2. DP voltammograms of *p*-cresol ($c = 1\times10^{-4}$ mol l^{-1}) in acidic part of Britton-Robinson buffer (pH = 1.9). Measured at boron doped diamond electrodes deposited at B/C ratio: (a) 500 ppm, (b) 1000 ppm, (c) 4000 ppm, and (d) 8000 ppm.

Table 1
The detection limits of *p*-cresol in acidic part of Britton-Robinson buffer (pH = 1.9), measured by selected voltammetric methods at BDD electrode with different boron concentration.

B/C ratio	Limits of detection [μmol l^{-1}]		
	DPV	DCV	SWV
500 ppm	0.45	0.57	–
1000 ppm	0.17	0.18	5.32
4000 ppm	0.06	0.09	4.39
8000 ppm	0.14	0.16	17.6

of conductivity and differential pulse voltammetry should be preferred for quantitation of *p*-cresol. The lowest limits of detection for all three electrochemical methods were achieved using electrode with B/C ratio 4000 ppm.

4. Conclusion

Well-shaped voltammetric peaks in acidic medium after anodic activation of BDD electrodes with different boron concentration were obtained for oxidation of *p*-cresol. Electrodes with metallic type of conductivity outperformed in analytical parameters the semi-conductive electrodes. Further effort will be devoted to the application of developed methods for analysis of real matrices including preliminary separation and preconcentration steps for differentiation of the individual cresol isomers.

Acknowledgments

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Electrochemical determination of 2-nitrofluorene and investigation of its interaction with DNA

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Keywords
DNA biosensor
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glassy carbon electrode
2-nitrofluorene
voltammetry

Abstract
2-Nitrofluorene (2-NF) belongs to the group of nitrated polycyclic aromatic hydrocarbons. These compounds are categorized as environmental pollutants and they can manifest mutagenic and carcinogenic effects. A differential pulse voltammetric method at a glassy carbon electrode (GCE) was developed for sensitive determination of 2-NF. Under the optimum conditions found, a linear calibration dependence was obtained in the concentration range of 2×10^{-7}–1×10^{-5} mol L^{-1}, with the limit of quantification of 2×10^{-7} mol L^{-1}. The practical applicability of this method was verified on the direct determination of 2-NF in a model sample of sand. Moreover, the mutual interaction between 2-NF and DNA was investigated using an electrochemical DNA biosensor (DNA-modified GCE). Cyclic voltammetry, electrochemical impedance spectroscopy, and square-wave voltammetry were employed in this study, confirming the formation of a 2-NF–DNA complex.

1. Introduction

2-Nitrofluorene (2-NF, Fig. 1) is produced by incomplete combustion of organic compounds, thus contaminating the living and working environment. It is dangerous because of its carcinogenicity and mutagenicity [1]. 2-NF is located in the air either free or adsorbed on solid particles, but also in water or soil. In human body, 2-NF is extensively

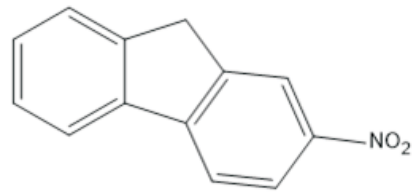

Fig. 1. Structure of 2-nitrofluorene.

metabolized (reduced, acetylated, and hydroxylated). The hydroxylated form of 2-NF exhibits mutagenic effects [2, 3].

Various electrochemical methods have been developed for sensitive determination of 2-NF, utilizing different types of working electrodes. As the very first one, dropping mercury electrode has been used for differential pulse polarographic determination of 2-NF, with the limit of quantification (*LOQ*) of 4×10^{-7} mol L^{-1} [4]. Differential pulse voltammetry (DPV) and differential pulse adsorptive stripping voltammetry (DPAdSV) at a hanging mercury drop electrode have also been used, with the *LOQ*s of 4×10^{-8} mol L^{-1} and 3×10^{-9} mol L^{-1}, respectively [4]. Recently, a mercury meniscus modified silver solid amalgam electrode (DPV and DPAdSV with the *LOQ*s of 2×10^{-7} mol L^{-1} and 2×10^{-9} mol L^{-1}, respectively) [5, 6], a polished silver solid amalgam composite electrode (DPV with the LOQ of 3×10^{-6} mol L^{-1}) [7], and a carbon paste electrode based on glassy-carbon microbeads (DPV with the *LOQ* of 8×10^{-6} mol L^{-1}) [5] have also been successfully applied. In addition, the interaction of 2-NF with DNA has been studied using an electrochemical DNA biosensors based on screen-printed carbon paste electrodes [8].

The aim of this work was firstly to develop a new, rapid, inexpensive, and sensitive method for DPV determination of 2-NF at a glassy carbon electrode (GCE). Secondly, the interaction of 2-NF with DNA was investigated using an electrochemical DNA biosensor prepared by adsorption of double-stranded DNA on the GCE surface.

2. Experimental

2.1 Reagents and chemicals

Stock solution of 2-NF ($c = 1\times10^{-3}$ mol L^{-1}) was prepared by dissolving 0.0211 g of 2-NF (98%, Sigma-Aldrich) in 100 mL of ethanol (99.8%, Lach-Ner, Czech Republic) using an ultrasonic bath. Phosphate buffer (PB, 0.1 mol L^{-1}, pH = 6.7) was prepared by dissolving 35.8 g $Na_2HPO_4.12H_2O$ (98.5%, Lach-Ner) and 13.8 g $NaH_2PO_4.H_2O$ (>98%, Sigma-Aldrich) in 1 L of deionized water. Britton-Robinson buffer (BRB) was prepared by mixing acidic (0.04 mol L^{-1}) and basic (0.2 mol L^{-1}) components. 1 L of the acidic component was prepared from 2.47 g H_3BO_3, 2.3 mL 98% CH_3COOH, and 2.5 mL 85% H_3PO_4 (all Merck); the basic component was prepared by dissolving 8.0 g NaOH (Merck) in 1 L deionized water. Redox indicator (a 1×10^{-3} mol L^{-1} equimolar mixture of $[Fe(CN)_6]^{4-}$ and $[Fe(CN)_6]^{3-}$ anions) was prepared by dissolving 0.2112 g $K_4[Fe(CN)_6].3H_2O$ and 0.1646 g $K_3[Fe(CN)_6]$ (both 99%, Lachema) in 500 mL of PB (pH = 6.7). The stock solution of DNA (γ_{DNA} = 10 mg mL^{-1} or 100 mg mL^{-1}) was prepared by dissolving 10 mg or 100 mg of low-molecular-weight salmon sperm double-stranded DNA (Sigma-Aldrich) in 1 mL of PB (pH = 6.7) and it was stored in a plastic Eppendorf tube in a freezer at 4 °C. Deionized water produced by a Milli-Q Plus system (Millipore, USA) was used.

All solutions were stored in glass bottles in dark at laboratory temperature, with the exception of DNA stock solutions.

2.2 Instrumentation and procedures

Differential pulse voltammetry (DPV, polarization rate of 20 mV s^{-1}, pulse height of 50 mV, pulse width of 100 ms, step potential of 5 mV, potential range from 200 to 1000 mV), cyclic voltammetry (CV, polarization rate of 50 mV s^{-1}, step potential of 5 mV, potential range from 0 to 500 mV), electrochemical impedance spectroscopy (EIS, polarization potential of 225 mV, potential amplitude of 10 mV, frequency range from 0.1 to 5000 Hz), and square-wave voltammetry (SWV, polarization rate of 3 V s^{-1}, potential amplitude of 40 mV, step potential of 15 mV, frequency of 200 Hz, potential range from 0 to 1600 mV) were carried out with a MicroAutolab III/FRA 2 (Eco Chemie, Netherlands) instrument. GPES (for DPV, CV, and SWV) and FRA (for EIS) software, version 4.9, were used. The MicroAutolab III/FRA 2 was controlled by a desktop computer running under Microsoft Windows XP Professional. Measurements were carried out in a three-electrode system with a glassy carbon working electrode (GCE, disc diameter of 3.0 mm, Metrohm), a Ag|AgCl (3 mol L^{-1} KCl) reference electrode (Monokrystaly, Czech Republic), and a platinum wire auxiliary electrode (Monokrystaly).

All measurements were carried out in a total volume of 10 mL. Oxygen was removed by bubbling with nitrogen for 5 min (purity class 4.0, Linde, Czech Republic) before each measurement. All measurements were carried out at laboratory temperature.

3. Results and discussion

3.1 Determination of 2-NF by DPV at the GCE

DPV at the GCE was used for the determination of submicromolar concentrations of 2-NF. The optimum pH of the used buffer was chosen as pH = 7.0, based on the results obtained from the pH dependence investigated using BRBs of various pH values (from 1.0 to 13.0) in a mixture of BRB:ethanol = 1:1 (*v/v*). The relative standard deviation of 20 repeated measurements of 1×10^{-4} mol L^{-1} 2-NF in BRB (pH = 7.0):ethanol = 1:1 (*v/v*) was 1.1% (the solution was stirred by bubbling with nitrogen for 15 s between each measurement). Under the optimum conditions found, the obtained calibration curve was linear in the concentration range of 2×10^{-7}–1×10^{-5} mol L^{-1}. The analytical parameters of calibration dependence are: the slope is –40.87 mA L mol^{-1}, the intercept is 5.34 nA, coefficient of determination is 1.000 and *LOQ* is 2×10^{-7} mol L^{-1}.

The practical applicability of the newly developed method was verified on the determination of 2-NF in model samples of sand (size of particles less than 1 mm) after a liquid extraction with ethanol (3 g of sand spiked with the appropriate

amount of the 2-NF stock solution were suspended in 3 mL of ethanol; then, 1 mL of the extract was mixed with 2 mL of ethanol and filled up to 10 mL with PB (pH = 6.7)). The extraction yields of 2-NF from the model samples of sand were measured by DPV at the GCE in the concentration range of 1×10^{-7}–1×10^{-5} mol L^{-1} (the GCE surface was polished before each change of the 2-NF concentration) in the solution of PB (pH = 6.7):ethanol = 7:3 (*v/v*). PB (pH = 6.7) was used instead of BRB (pH = 7.0) for simplification. The extraction yields of 2-NF ranged from 94.5 to 96.5%.

3.2 Preparation of the DNA biosensor

Optimal conditions for the preparation of the DNA biosensor for CV and EIS measurements were determined by CV and EIS: the DNA biosensor was prepared by accumulation of DNA on the GCE surface (accumulation time of 2 min) from the 10 mg mL^{-1} DNA solution in PB (pH = 6.7). Optimal conditions for the preparation of the DNA biosensor for SWV measurements were determined by SWV: as optimal parameters of DNA accumulation were chosen the accumulation time of 30 s and the concentration of DNA in the accumulation solution of 1 mg mL^{-1}.

3.3 Investigation of the interaction of 2-NF with DNA by CV, EIS, and SWV at the DNA biosensor

Well-developed peaks of 2-NF, corresponding to the reduction of the nitro group, can be seen using CV at the unmodified GCE (Fig. 2, curve 1) as well as at the DNA-GCE (Fig. 2, curve 2). DNA accumulated on the GCE made the access of 2-NF to its surface more difficult (lower peak with its potential shifted to more negative values was observed). Moreover, CV showed the ability of 2-NF to bind to DNA (to form a 2-NFDNA complex) during a 5 min incubation of the DNA biosensor in the solution of 1×10^{-4} mol L^{-1} 2-NF (Fig. 2, curves 3 and 4).

EIS technique did not reveal any significant change in the electrochemical behavior of the DNA biosensor before and after a 5 min incubation in the solution of 1×10^{-4} mol L^{-1} 2-NF in PB (pH 6.7):ethanol 1:1 (*v/v*). Nyquist plots were measured in the solution of the redox indicator, 1×10^{-3} mol L^{-1} $[Fe(CN)_6]^{4-/3-}$ in PB (pH = 6.7). We can assume that the above-mentioned formation of the 2-NFDNA complex did not induce any DNA double-strand breaks (resulting in a release of DNA fragments from the biosensor surface).

The dependence of the relative biosensor response to damage of DNA accumulated on the DNA-GCE surface on the time of the incubation in the solution of 1×10^{-5} mol L^{-1} 2-NF in PB (pH = 6.7), which was measured using SWV, showed decreasing peaks of guanosine and adenosine moieties present in the DNA structure with the increasing incubation time, thus confirming the DNA damaging effect of 2-NF (probably induced by changes in the DNA structure leading to reduction of the number of DNA electroactive sites).

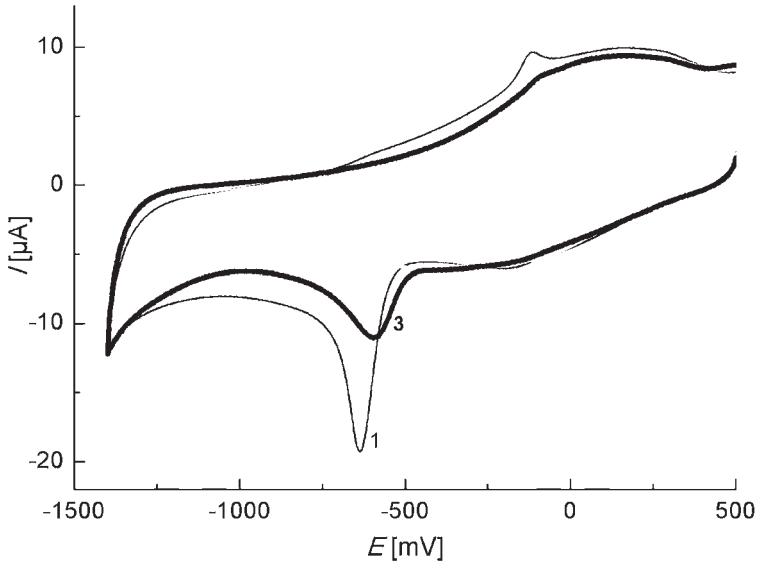

Fig. 1. Cyclic voltammograms obtained in the solution of 1×10^{-4} mol L^{-1} 2-NF in PB (pH = 6.7) with ethanol 1:1 (*v/v*) on the unmodified GCE (curve 1) and on the DNA biosensor (curve 2); cyclic voltammograms obtained in PB (pH = 6.7) before (curve 4) and after a 5 min incubation (curve 3) of the DNA biosensor in the solution of 1×10^{-4} mol L^{-1} 2-NF in PB (pH = 6.7):ethanol 1:1 (v/v).

4. Conclusions

A rapid, simple, and sensitive DPV method for determination of 2-NF has been developed. The calibration curve of 2-NF measured in the optimum medium of BRB:ethanol = 1:1 (*v/v*) was linear in the concentration range from 2×10^{-7} to 1×10^{-5} mol L^{-1}, with the *LOQ* of 0.2 μmol L^{-1}. The applicability of the method was tested on the model samples of sand after the liquid extraction with ethanol, with the added/found recoveries from 94.5 to 96.5% obtained within the concentration range from 1×10^{-7} to 1×10^{-5} mol L^{-1}. These results demonstrate the practical applicability of the developed method for sensitive determination of 2-NF in simple solid environmental matrices.

Moreover, rapid and inexpensive methods for investigation of the interaction of 2-NF with DNA were applied, utilizing an electrochemical DNA biosensors based on a GCE. CV showed that 2-NF binds to DNA, forming a 2-NFDNA complex, whereas no significant changes in electrochemical characteristics of the biosensor after its incubation with 2-NF were observed using EIS. In addition, SWV indicated a DNA damaging effect of 2-NF.

Acknowledgments

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Determination of As(III) using UV-photochemical generation of its volatile compounds and QF-AAS in flow injection mode employing Sb(III) as reaction modifier

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Keywords
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Abstract
The work describes the development, optimization and characterisation of an analytical method for determination of As(III) in model aqueous solutions employing its UV-photochemical volatile compounds generation and atomic absorption spectrometry utilizing an externally heated quartz furnace as an atomizer in flow injection arrangement. Formic acid of 2.75 mol L^{-1} acted as the photochemical agent during the UV-assisted conversion of As(III) from the solution to the gaseous phase. Moreover, the study investigated the influence of selected compounds on the signal measured with the aim to improve sensitivity of As(III) determination. Sb(III) was found as the most suitable reaction modifier. The concentration of 3 mg L^{-1} Sb(III) increases the absorbance of arsenic approximately sixty times compared to signals attained without any modification.

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]:

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

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

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

It is necessary that the products formed from arsenite 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

Deionized water prepared in a MilliQplus system (18.2 MΩ cm, Millipore) was used for dilution of all the solutions. The stock solution of As(III) was prepared by dissolving of the appropriate amount of arsenic trioxide (> 99.5%, Sigma-Aldrich) in slightly alkaline (solid KOH, 89.0%, Lach-Ner, CZ) solution. Formic acid (≥ 98%, Sigma-Aldrich) was used as UV-photochemical reaction agent and its solutions were prepared fresh daily. Argon (99.998%; Linde Gas, CZ) was used as the inert carrier gas during all the experiments. Hydrogen (99.998%; Linde Gas, CZ) was used as the reaction gas during all the experiments. The solutions of the chemical modifier were prepared from the standard solution of Sb(III) (1000±2 mg dm^{-3}, Merck).

2.2 Instrumentation

- Atomic absorption spectrometer ContrAA700 (Analytik Jena, DEU)
- Programmable peristaltic pump Masterflex (Cole-Parmer, USA)
- Power supply for the UV-generator Modus SB 18 (Modus s.r.o., 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 3× (Cole-Parmer, USA)
- Six-way injection valve FIA V-451 (Upchurch Scientific, USA) with 100 µl sampling loop
- Syringe size 10 ml (Hamilton, USA)

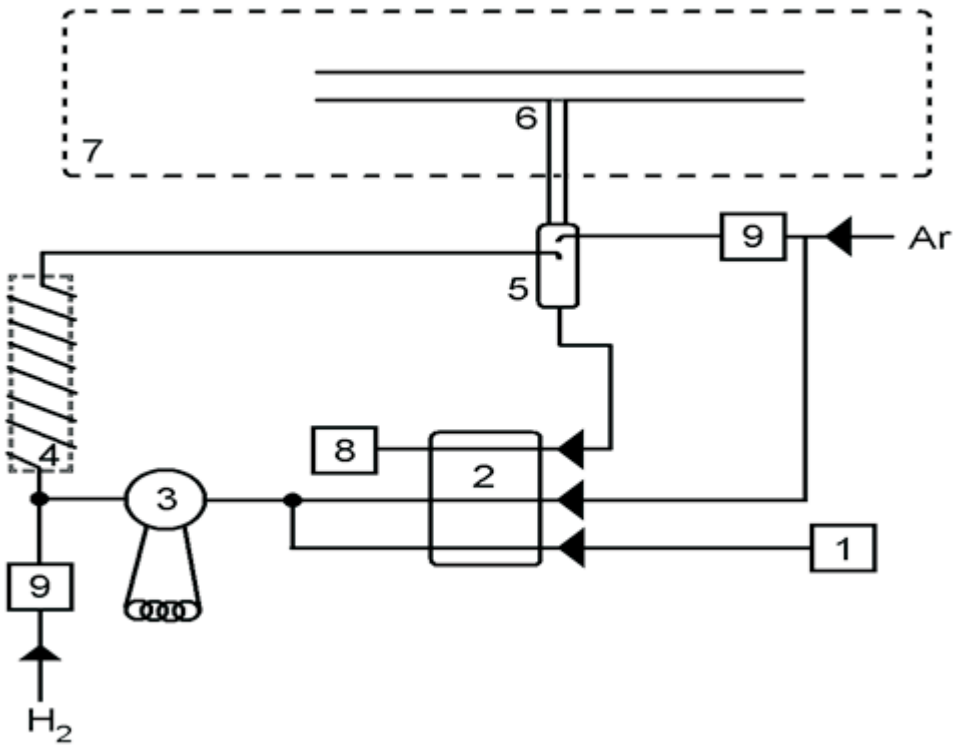

Fig. 1. The instrumental set-up: (1) reservoir bottle with solution of HCOOH, (2) peristaltic pump, (3) six-way injection valve, (4) reaction coil with UV-generator, (5) gas-liquid separator, (6) atomizer, (7) AAS, (8) waste bottle, (9) gas flow controller.

- 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

The relevant parameters influencing the efficiency of UV-photochemical volatile compounds generation have already been optimized in the work [13] but they were verified for this study. The optimum experimental conditions found for UV-PVG are recapitulated in Table 1.

3.1 Carrier gas (argon) flow rate

There were used two ways of input of inert gas (argon) into the apparatus. The first inlet was placed prior the six-way injection valve. This kind of carrier gas introduction was used for segmentation of carrier liquid and for prevention of spread zones of the injected sample. The highest absorbance was obtained for

Table 1
Optimum condition.

Parameter	Value
HCOOH concentration	2.75 mol L^{-1}
H_2 flow rate	30 mL min^{-1}
Ar flow rate (gas-liquid separator)	30 mL min^{-1}
Ar flow rate	50 mL min^{-1}
Sampling loop volume	300 μL
Carrier flow rate	3.0 mL min^{-1}
Reaction coil length	251 cm
Atomization temperature	950 °C

flow rate of 50 mL min^{-1} of argon. Secondly, argon was introduced into the gas-liquid separator. Its presence was necessary for release of the arsenic volatile compounds from the liquid phase and for their transport to the atomizer. The best analytical signal was provided by 30 mL min^{-1} of carrier gas (argon).

3.2 Flow rate of hydrogen

The introduction of hydrogen plays important role during UV-photochemical generation of volatile compounds and the atomization of volatile compounds in an externally heated quartz tube atomizer. Therefore, it was investigated if added hydrogen can increased the signal. It was found that no volatile product containing arsenic was formed without hydrogen addition prior the UV-photoreactor. The signal increased with increasing amount of hydrogen up to 30 mL min^{-1}. Hydrogen was added to the optimum argon flow which was positioned before the UV-photochemical generator. The range of 0 to 65 mL min^{-1} of hydrogen was tested. 30 mL min^{-1} of hydrogen was selected as the optimum hydrogen flow rate.

3.3 Dependence of the absorbance on concentration of formic acid

Aqueous formic acid solution served as carrier liquid in this analytical method. Formic acid was selected as a UV-photochemical agent which was active during the reduction of As(III) ions to volatile compounds because it is a source of radicals. The HCOOH concentration was varied from 0 to 3.5 mol L^{-1}. The highest signals were observed for formic acid concentration of 2.75 mol L^{-1} as it can be seen in Fig. 2. The summary of optimum conditions is reported in Table 1.

3.4 Reaction modifiers

The study investigated the influence of selected compounds on the signal measured with the aim to improve sensitivity of As(III) determination. Bi(III) and Sb(III) were found as the most suitable reaction modifiers. Whereas Bi(III) at

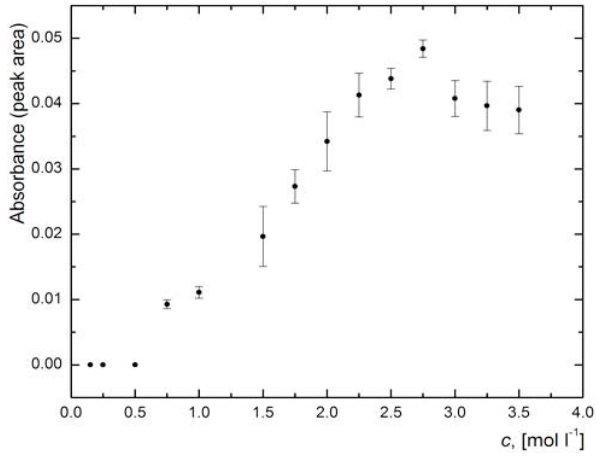

Fig. 2. Effect of concentration of formic acid. Concentration of arsenic: 1 mg L^{-1}. Other experimental conditions are given in Table 1.

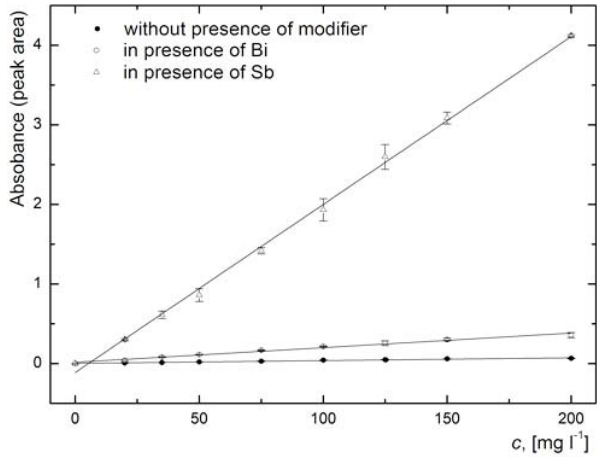

Fig. 3. Calibration curves of As(III) without and in presence of 5 mg L^{-1} Bi(III) and in presence of 3 mg L^{-1}Sb(III). Other experimental conditions are given in Table 1.

concentration of 5 mg L^{-1} increased the slope of the calibration of arsenic approximately six times compared to signals without reaction modifier, 3 mg L^{-1} of Sb(III) increased the sensitivity sixty times in the same way. Appropriate concentration of these two chemical modifiers was added to the solution of HCOOH before the measuring. Fig. 3 shows linear part of the calibration curves without or in presence of Bi (III) and without or in presence of Sb(III) ion respectively. A comparison of figures of merit is presented in Table 2. The limits of detection and quantification moved to the lower concentration level in presence of the reaction modifier.

Table 2
Figures of merit.

	Without presence of modifier	In presence of Bi(III)	In presence of Sb(III)
LOD [μg L^{-1}]	2.64	0.42	0.048
LOQ [μg L^{-1}]	8.81	1.4	0.16
Sensitivity [L μg^{-1}]	4×10^{-4}	21×10^{-4}	21×10^{-3}
Repeatability [RSD %]	10.2	20.3	17.7
R	0.994	0.995	0.995

4. Conclusions

An analytical method for determination of arsenic using its UV-photochemical volatile compounds generation was adjusted 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. Bi(III) increased the absorbance of arsenic almost eleven times as it was found in the previous study by the activation. This reaction modifier was not added into the sample or to the carrier liquid [13]. On the other hand, Sb (III) at concentration of 3 mg L^{-1} significantly increased the absorbance of arsenic almost sixty times but its effect did not persist for the rest of the day like with Bi (III) ions. The proposed method is distinguished by a detection limit of 0.048 μg L^{-1} of arsenic, by a sensitivity of 21×10^{-3} L μg^{-1}, by a repeatability of 17.7% under the optimum conditions with the addition of Sb(III) ions.

Acknowledgments

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Identification of pollutants at the adjacent areas to the landfill site using two dimensional gas chromatography with mass spectrometry technique

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Keywords
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Abstract
Municipal landfills are considered one of the main sources of air pollutants. In this article is presented the use of a modern analytical technique: a two-dimensional gas chromatography to identify the substances in the atmospheric air near a waste processing plant located in Gdansk. In order to determine the types of substances occurring in different atmospheric conditions, the study was conducted from February to April 2016. Based on the obtained results, the most common chemical substances were identified and listed with respect to their impact on the environment and human health.

1. Introduction

Landfills are considered the main source of air pollution. Huge amounts of substances, which are the product of bio-chemical waste processing: composting, incineration, disposal, recycling are produced. Currently disposal is the most common method of waste management in many regions of the world [1]. The most elementary issue is the large area of emissions of toxic and malodorous substances. Landfill gas is produced during decomposition of organic matter by aerobic and anaerobic microorganisms [2]. Carbon dioxide and methane are the main components of biogas. Both of these gases are believed to be the cause of the greenhouse effect. Moreover, methane is also dangerous due to its explosive properties [3]. It is important to note the fact that many other substances are released into the atmosphere. They can be divided into two groups: the odors and toxic chemicals. The odors are organic and inorganic chemicals detected by the

olfactory receptors at very low concentrations and perceived as unpleasant, for instance hydrogen sulfide, mercaptans, ammonia, dimethyl sulfide, trimethylamine. The nuisance odors are a universal problem for people living in the vicinity of landfills [4, 5]. They complain more and more about lowering quality of their life. Some of emitted substances can be dangerous to human health. The way the chemicals affect human being depends on the concentration, exposure time and substances type, e.g.: aromatic hydrocarbons (benzene, toluene) are carcinogenic, carbon monoxide is inorganic substances causes anoxia.

The aim of the studies was to identify the substances present in atmospheric air in various weather conditions. Furthermore, performed investigation could be a basis to air quality assessment. The results obtained could be an impulse for more detailed study on compounds identified during the investigations and their impact on human health.

2. Experimental

2.1 Sampling

The samples of atmospheric air were collected near a waste processing plant located in Gdansk, from February to April. The locations of sampling are presented in Fig. 1. A device called Gas Sampling System (GSS), specially designed by Gerstel

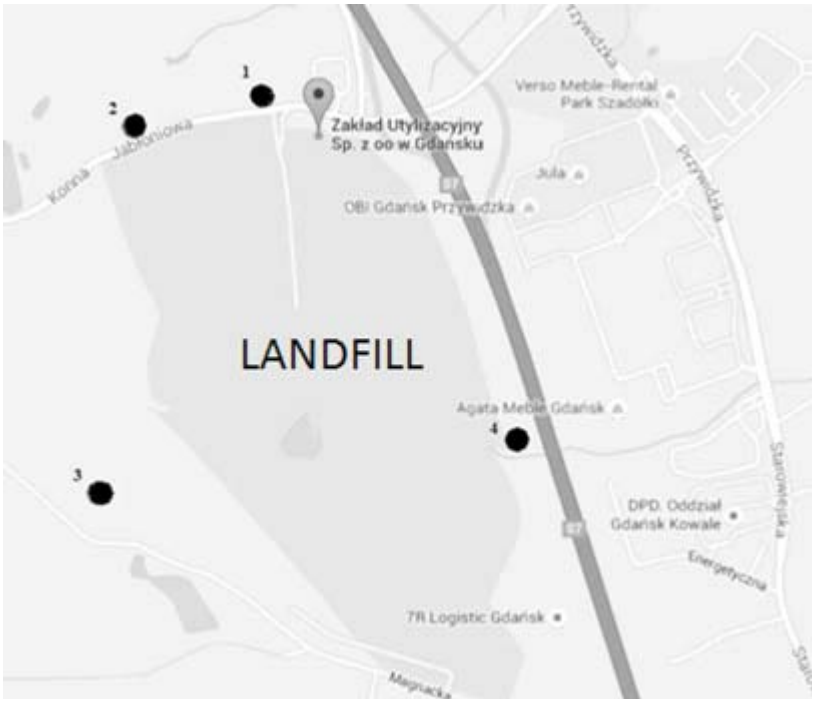

Fig. 1. Sampling locations.

Table 1
The parameters of device during analysis of air samples.

Section	Parameter
Columns	Two capillary columns: ▪ initial oven: 30 m×250 μm×0.25 μm, sorbent: Equity 1 ▪ secondary oven 2 m×100 μm×0.10 μm, sorbent: SGWAX
Carrier gas	helium, flow rate 1.0 ml min^{-1}
Temperature program	▪ initial temperature 40 °C ▪ holding time 1min ▪ gradient 4 °C/min ▪ target temperature 190 °C ▪ holding time 0 min ▪ gradient 10 °C/min ▪ final temperature 240 °C ▪ holding time 1 min
Modulation time	5 s
Mass spectrometer parameters	▪ ion mass range 15–500 [u] ▪ detector voltage 1600 [V] ▪ electron impact mode –70[V] ▪ ion source temperature 250 °C

company (Germany), was used for the sampling of gas. The sampling relies on the passing a stream of air (at a flow rate of 75 ml/min over a period of 30 minutes) through tubes filled with a solid sorbent poly(oxy-2,6-diphenyl-1,4-phenyl) (trade name:Tenax TA) placed in special channel. The device is equipped with two channels, so that samples can be taken individually for each of them, or both at the same time. Prior to performing the procedure described above, each tube was subjected to thermal desorption at 300 °C to remove potential contaminants (from previous studies) from the sorbent.

2.2 Instrumentation

The analysis of samples was conducted using two-dimensional gas chromatography with time-of-flight mass spectrometry (GC×GC-TOFMS) technique by equipment illustrated in Fig. 2. The time of analysis of a single sample was 60 minutes. The device parameters during analysis of air samples are presented in Table 1.

3. Results and discussion

The investigations revealed that many types of substances are present in the atmospheric air, including: aromatic hydrocarbons, alkanes, alkenes, ketones, aldehydes, ammonia, nitriles alcohols, oxygenated aromatic compounds, terpenes. Some of them are presented in Fig. 2 as 3D chromatogram. Weather conditions and the most common substances in particular months are presented

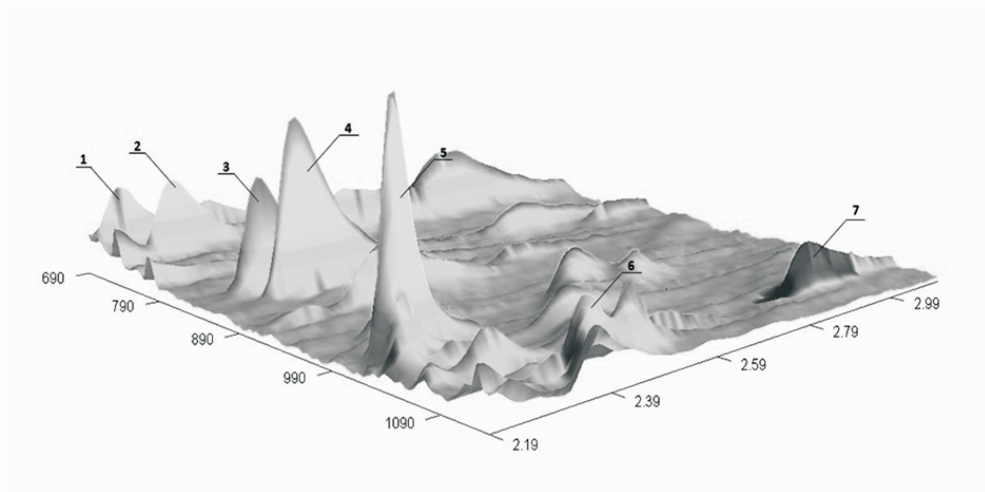

Fig. 2. 3D chromatogram of an air sample. The numbers indicate particular chemical compounds: (1) ethylbutyric acid, (2) acetic acid butylester, (3) ethylbenzene, (4) 1,3-dimethylbenzene, (5) dodecane, (6) tetradecane, (7) nonan.

in Table 2. In all samples were dominated by compounds from the group of hydrocarbons (aliphatic and aromatic). The most common substances among samples collected in February were benzene and derivatives. This chemical compounds are called BTEX (benzene, toluene, ethylobenzene, xylene). The main source of BTEX emission is combustion of fuels [6]. Some of the samples were taken with heavy traffic of motor vehicles, which could be the cause of the high level of BTEX. These chemicals can be responsible for eye and skin irritations [7]. Moreover,

Table 2
Weather conditions and identified substances in individual months [9].

Month	Temperature [°C]			Humidity [%]			Wind speed [m/s]			Wind direction (dominant)	Compounds
	max	min	mid	max	min	mid	max	min	mid		
February	9.12	–3.31	2.5	96.41	64.55	83	20.30	0.00	6.00	northwest	benzene toluene heksane α-pinen terpinene xylene
March	15.75	–1.04	5.5	96.50	46.96	79	12.20	0.00	5.00	southeast	benzene α-pinen terpinene limonene sabinene
April	20.70	0.83	8.0	96.41	33.88	68	17.30	0.00	7.00	southwest	dodecane benzene

benzene is highly carcinogenic. Terpinen and α-pinene were also identified in almost all of the samples. They are the substances belonging to terpenes group, which are known for their strong fragrance [8]. The air conditions from February to April were mostly consistent, so it is not possible to draw conclusions regarding whether the presence of particular substances in atmospheric air is dependent on these conditions.

4. Conclusions

The aim of this investigation was the identification of as many volatile compounds present in the atmospheric air as possible. This goal was achieved. Based on the results it can be concluded, that two-dimensional gas chromatography is a useful and effective technique for identifying chemicals in atmospheric air. However, in order to determine the dependence between air conditions and the type of substances in atmospheric air, more detailed studies are needed, i.e. the investigations should be carried out throughout the year.

Acknowledgments

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Comprehensive two dimensional gas chromatography coupled with mass spectrometry as tool for characterization of the main pollutants present in atmospheric air in Gdansk

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Keywords
industrial zones
pollutants in atmospheric air
polycyclic aromatic hydrocarbons
two-dimensional gas chromatography
volatile organic compounds

Abstract
Nowadays, the technique that is commonly used to identify and quantify the chemical compounds in gas sample is gas chromatography. The separation resolution in gas chromatography can be improved by applying the modulation technique. In this paper presented is the most important information regarding the pollutants present in atmospheric air. The areas in which the tests were carried out are one of the largest industrial zones in Gdansk. The research was aimed at identifying the most common chemical compounds. The Comprehensive Two-Dimensional Gas Chromatography with Time-of-Flight Mass Spectrometry (GC×GC-TOFMS) technique was applied for the analysis of volatile organic compounds in samples of atmospheric air. Field studies were conducted from March to May 2016.

1. Introduction

Chromatography is one of the techniques used to separate the different components of mixtures and determine their chemical composition. This is possible due to the varying strength of interactions between the components of the mixture and the stationary and mobile phases. Gas chromatography coupled with mass spectrometry allows for separation and identification of chemical components with different properties.

Gas chromatography is used to evaluate the parameters of the environment and the degree of chemical contamination present in the air, soil, surface water and groundwater. One of the main sources of emission are industrial areas, mainly

from the oil, food, cosmetic, pharmaceutical, and automotive industries. Polycyclic aromatic hydrocarbons, volatile organic compounds, pesticides, dioxins, furans, alkylphenols, and phthalates are the most frequently identified chemicals [1]. Conventional one-dimensional gas chromatography is limited in the amount of the compounds that can be analyzed; app. 150 at the same time [2]. However, when the samples contain chemical substances with similar properties it is often insufficient. Therefore, increasingly popular is the two-dimensional chromatography technique. Analysis using this technique is more complicated, but the separation efficiency is much greater [3–5].

Some of the compounds present in the samples may adversely affect humans, animals, plants and other elements of the environment. Air pollution is the most dangerous form of pollution, because it is mobile and can contaminate large areas [6]. Exposure to air pollutants increases the risk of cancer, respiratory diseases and allergies. The air in Poland is one of the most polluted in Europe. The aim of the study was to determine the effect of the proximity of industrial plants on air quality in Gdansk. Presented are the identified chemical compounds. The dangers associated with the impact of the most dangerous substances on human health are also discussed [7].

2. Experimental

2.1 Sampling

The samples of atmospheric air were collected in the areas adjacent to one of the biggest industrial plants in Gdansk. These were: Lotos Group refinery, "Fosfory" phosphate fertilizers company, municipal landfill located in Gdansk Szadolki, and wastewater treatment plant "Gdansk-Wschod". The activities of these companies are connected with large emission of pollutants into the air, affecting its quality. Presented in Fig. 1 is a map with location of measurement points where the samples were taken. The air was sampled from March till May 2016.

Fig. 1. Sampling areas in Gdansk.

Table 1
GC-temperature program.

	Primary oven	Secondary oven
Temperature	40 °C, incubated for 1 min	45 °C, incubated for 1 min
Gradient temperature I	10 °C/min till 90 °C	10 °C/min till 95 °C
Gradient temperature II	3 °C/min till 240 °C (maintain temperature for 5min)	3 °C/min till 245 °C (maintain temperature for 5min)

Table 2
GC-column characteristic.

	Column I	Column II
Type	capillary column	capillary column
Length	30 m	2 m
Inside diameter	250 µm	100 µm
Maximum temperature	325 °C	280 °C
Thickness of film of stationary phase	0.25 µm (trade name: Equity1)	0.1 µm (trade name: SGWAX)

The air samples were collected using the device called Gas Sampling System (GSS) manufactured by GERSTEL company. During the study, the tubes filled with solid sorbent: poly(oxy-2,6-diphenyl-1,4-phenyl), trade name: Tenax TA. They were placed in the device which was pumping atmospheric air through this tubes in order to collect the chemical compounds on the sorbent. Before sampling, each tube was subjected to the thermal desorption at 300 °C, to eliminate the leftover compounds and other contaminants which remaining on the sorbent after the previous analysis. The volumetric flow rate was 75 mL/min. Sampling time for analysis was constant and for each tube it was 30 minutes. After this time the tubes were stored in sealed, dedicated containers at app. 15 °C.

2.2 Instrumentation

The Comprehensive Two-Dimensional Gas Chromatography with Time-of-Flight Mass Spectrometer device was used to analyze the samples. The time of a single analysis was 60 minutes. Conditions during each analyses are presented in Tables 1 and 2.

3. Results and discussion

Presented in Table 3 are the identified chemical compounds from one of the air samples gathered in Gdansk. It was discovered that the largest group of chemical compounds were the arenas and alkanes. The presence of these compounds in

Table 3
The chemical compounds identified in the sample of atmospheric air.

Name	Retention time [s]	Probability	Peak area	Atomic mass unit	*S/N*
acetaldehyde	310	994	3504256	43	7008.4
ethanol	320	973	36630672	45	88192
2-nitrobutane	320	807	99260	57	1452.1
pentane	340	927	6038672	41	14821
hexane	385	951	3335778	57	18575
benzene	425	974	11344733	78	70154
1-hexene	430	836	820858	56	5049.3
m-xylene	645	966	2896171	91	13857
o-xylene	680	962	798742	91	5061.1
benzaldehyde	765	971	1757406	105	4171.4
camphene	790	909	33547	93	472.29
sabinene	825	933	423139	93	6517.6
α-pinene	840	937	802778	93	9702.3
dl-limonene	935	946	2468534	68	18569
acetophenone	965	964	1321371	77	2209
terpinene	990	868	175400	93	1533.3
nonanal	1055	936	298191	57	1353.2
dodecane	1330	953	522399	57	3524.9
fenylooctan	1960	899	108767	92	889
pentadecane	2060	951	256980	57	2781.1
hexadecanal	2275	934	157820	57	1265.1
methyl dihydrojasmonate	2330	927	578794	83	2990
ethyl 1-(4-isopropyl-phenyl) -2-methylpropyl	2410	882	300290	43	941.93
hexadecanoic acid	2995	919	605014	60	1422.6
geranylgeraniol	3505	849	24002136	69	13537

increased quantity can be caused by evaporation of petroleum products and during other processes that take place in industrial plants [8]. In each of examined air samples the compounds from the group of terpenes, aldehydes and esters were also present.

Benzene, toluene, ethylbenzene, and xylenes (BTEX) are aromatic hydrocarbons, that quickly evaporate when released into the air. Benzene is highly flammable and it is irritant to the skin and eyes. It can cause damage of the upper respiratory tract, cancer and heritable genetic damage [9]. Toluene is also flammable and can damage the air passages, cause dizziness and have negative influence on the fetus development [10]. Xylene isomers have similar characteristics to each other; mainly they are harmful to the skin and respiratory system [9].

Terpenes are chemical compounds with the general form $(C_5H_8)_n$, mostly phytochemicals. They include: limonene, terpinene, α-pinene, camphene, and geraniol. Limonene is a chemical compound which in contact with the skin can cause a strong allergic reaction. It can damage upper respiratory tract [9].

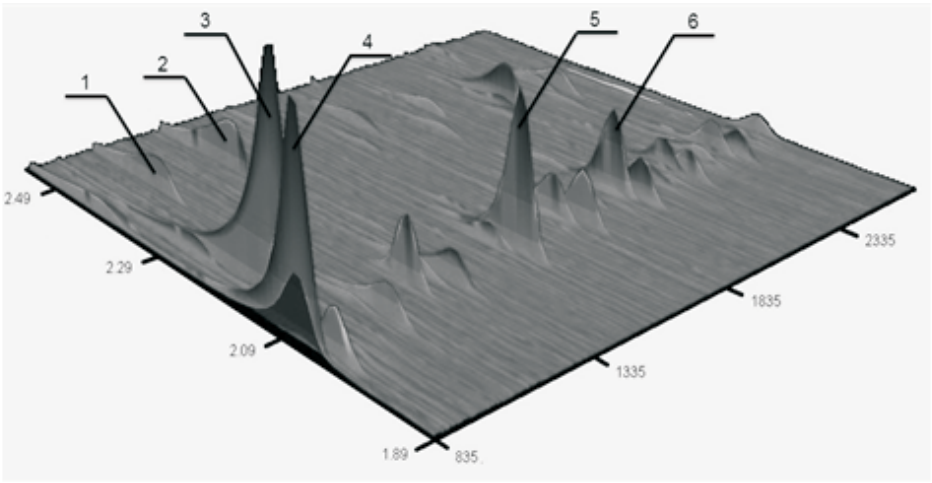

Fig. 2. The chromatogram obtained by three dimensional analysis: (1) nonanal, (2) decanal, (3) DL-limonene, (4) α-pinene, (5) tetradecane, (6) Tsete.

Presented in Fig. 2 is an enlarged fragment of a chromatogram obtained during one of the analysis.

4. Conclusions

The most dangerous and toxic are the pollutants resulting from combustion of fossil fuels in engines. Two-dimensional gas chromatography was used to identify the most important pollutants present in the atmospheric air in Gdansk. As a result of field studies it was possible to identify numerous chemical compounds which are part of the air pollution and have negative effect on the environment. The most common group of chemical compounds was arenes, alkenes, and terpenes. The conducted research allowed to briefly examine the air quality in Gdansk. Air quality should be monitored, especially in the industrial areas. A number of factors may have influenced the results of field studies. In order to gather the most representative data in future research it is necessary to take into the account the weather conditions, mainly temperature, air humidity, and strength and direction of the wind.

Acknowledgments

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Characterization of a laboratory-constructed miniaturized device for fast CE-MS measurements

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Keywords
capillary electrophoresis
high throughput analysis
mass spectrometry
fast separation

Abstract
In the recent years, attention for fast separation of all kind of samples in combination with automated systems has increased. On that account we designed and constructed a miniaturized capillary electrophoresis system, which can achieve fast separations, due to short capillary length and high electric field strength. An integrated and exchangeable autosampler unit, which can be loaded with 19 samples at once, allowed high throughput measurements with small sample volumes down to 30 µL. The aim of this work was the analytical characterization of this device in combination with mass spectrometry. Hydrodynamic and electrokinetic injections were carried out with different injection parameters. Amino acids were used as model systems, to test the performance of the device, concerning speed of separation, precision, and sample throughput.

1. Introduction

The fast separation of biological, pharmaceutical, and clinical samples with high throughput is in demand. Capillary electrophoresis (CE) is a versatile and often used technique in instrumental analysis and many investigations with this separation method have been demonstrated in recent years [1, 2]. There is a continuing trend towards miniaturized portable devices, which enable an automated operation [3]. Therefore, we designed and constructed a miniaturized CE device, which can be hyphenated to mass spectrometry (MS) via an electrospray interface (ESI) [4]. Mass spectrometry is a versatile detection technique, with typical detection limits in the upper nanomolar concentration range [5]. The aim of this work was the effective coupling of the miniaturized CE unit with MS using short capillaries and applying high field strengths to enable high-throughput CE-MS measurements. In this work we present the analytical characterization of our laboratory constructed device for CE-MS measurements.

2. Experimental

2.1 Reagents and chemicals

Lysine, histidine, and arginine were purchased from Sigma-Aldrich. Formic acid and isopropanol were purchased from Merck. The amino acids were prepared as stock solutions (50 mM) and diluted with Millipore water to 100 µM before the experiment. The background electrolyte (BGE) consisted of 0.8 M formic acid with a pH of 1.8. A sheath liquid consisting of Millipore water, isopropanol, and formic acid (49.9:49.9:0.2, *v/v/v*) was used. Each solution was filtered with 0.2 µm PTFE Rotalibo®- syringe filters before use. All solutions were stored in the fridge at 16 °C before use.

2.2 Instrumentation

The CE device, which was used in this work was designed and developed in our group. The portable and miniaturized device is shown in Fig. 1. The box consists of PVC plates (12×12×26 cm) and has a weight of 5 kg. The PVC plates were fixed with Nylon screws. The frontside is covered with a Plexiglas board. The lower part (A) accommodates electronics and the motors for the turntable (B). A polished syringe needle (C) serves as HV electrode. The power supply (D), delivering up to

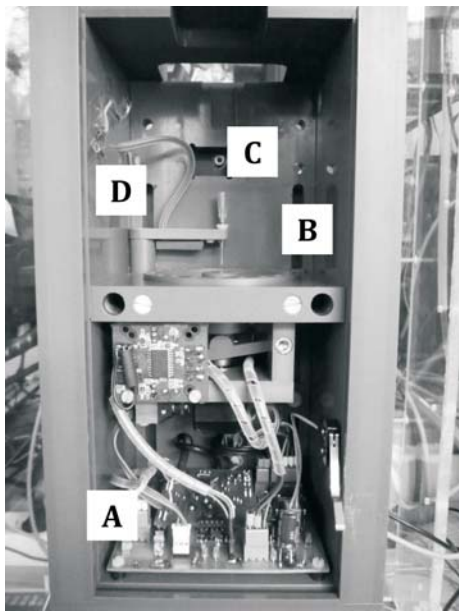

Fig. 1. Frontview of the CE device: (A) electronics for device controlling, (B) turntable, (C) HV-electrode, (D) HV-supply cable.

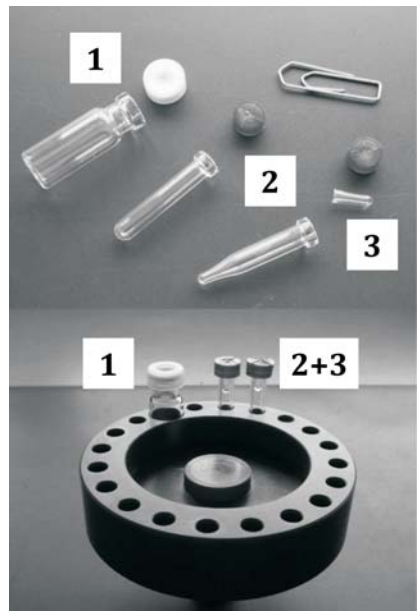

Fig. 2. (1) BGE vial (2 mL) with septum, (2) sample vial (0.3 mL, cylindric- and cone-shaped) with septum, (3) micro insert (30 µL).

30 kV was a model ESEG (Spezialelektronik GmbH, Germany). The CE device was controlled with a labview-based software. As reservoir for the BGE, a 2 mL vial (1) with septum from infochroma ag (Switzerland) was used. The vials and the sample tray are shown in Fig. 2. The sample vials were 0.3 mL glass vials (2) from Macherey-Nagel (Germany) with septum. Laboratory constructed micro inserts (3), which were made from 0.3 mL vials from VWR international (Germany) by cutting these vials at a suitable height, were used to obtain a minimum sample cavity of 30 µL. The capillaries for the CE measurements were purchased from Polymicro Technologies (USA) and had an inner diameter of 25 µm, an outer diameter of 360 µm, and a length of 25 cm. For conditioning they were flushed ten minutes with 0.1 M NaOH, ten minutes with Millipore water and 25 minutes with the BGE before use. For CE-MS experiments a Bruker micrOTOF (Bruker Daltonics) time-of-flight mass spectrometer was used. The mass analyzer enables a detection of ions in a mass range of 50–3000 *m/z*. The data acquisition was made with a Bruker software (micrOTOF control Version 2.3). A coaxial sheath liquid sprayer interface (Agilent) was used to couple the CE system with mass spectrometry. The sheath liquid was feed to the interface by a syringe pump from kd Scientific (USA).

3. Results and discussion

In order to find the best injection conditions, the relevant parameters were studied in detail. Hydrodynamic injections were made as well as electrokinetic injections. Injection time, injection voltage (depending on injection mode), and separation voltage were investigated. In case of electrokinetic injections short injection times and low injection voltages seemed to be the most important parameter settings, which led to a very good separation efficiency. Short injection times are also requested in the hydrodynamic injection mode to guarantee high separation efficiency. The separation voltage was set as high as possible to obtain fast separations, but low enough to prevent sparking. Fig. 3 shows an extracted ion electropherogram with optimized parameters. The separation of the three basic amino acids could be done in only 30 seconds. Repeated measurements, with the same experimental parameters, were made to evaluate the precision of the device. All 19 sample cavities of the sample tray were loaded with the same mixture of the model system. For repeated measurements (n = 3) relative standard deviations between 9% and 13% for the peak areas were obtained for the three model analytes (Table 1). The analytical performance was suitable to use this CE device for high-throughput CE-MS measurements. During repeated experiments a shift of the migration times was observed, which was due to the rather small BGE cavity of 100 µL. This problem was assigned to changes of the BGE caused by electrolysis during the electrophoretic separations. The sample tray was modified and a larger BGE vial (2 mL) was used, to overcome this drawback. With the new tray 100 separations could be performed without a significant shift of the migration times.

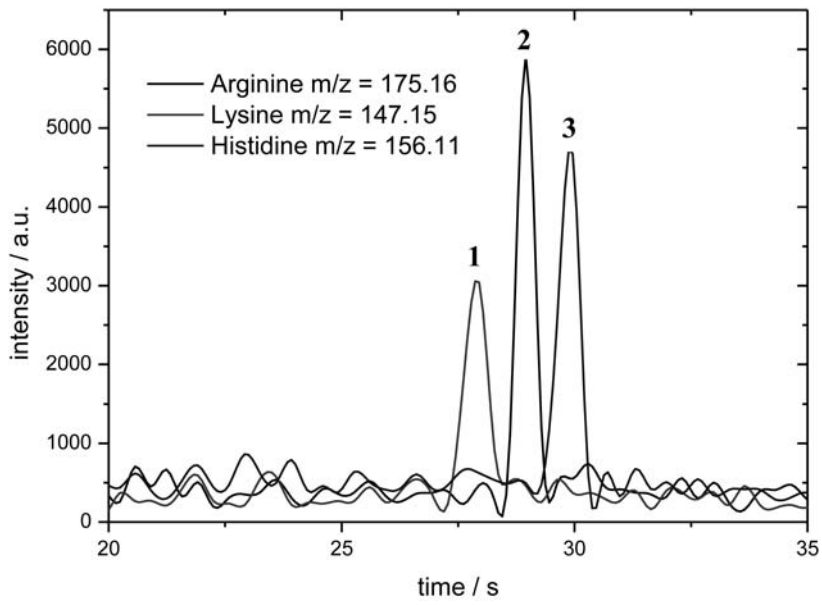

Fig. 3. Extracted ion electropherogram of a measurement under optimized conditions of (1) lysine, (2) arginine, and (3) histidine. Concentration: 100 μM, injection voltage: 4 kV, injection time: 1 s, capillary: l = 25 cm, ID = 25 μm, separation voltage: 30 kV, BGE: 0.8 M formic acid.

Table 1
Evaluation of the peak areas and the migration times for repeated experiments. Concentration: 100 μM, capillary: l = 25 cm, ID = 25 μm, separation voltage: 25 kV, hydrodynamic injection: 5 s, n = 3.

Compound	RSD of peak area [%]	RSD of migration time [%]
Lysine	10	5
Arginine	9	5
Histidine	13	5

4. Conclusion

In this work we presented a laboratory-constructed, miniaturized, and portable device for fast CE-MS measurements. Due to the short capillary length (25 cm) and the high electric field strength (1.2 kV/cm) a separation of a model system of amino acids could be done in 30 seconds. The migration times were much shorter in comparison to traditional CE systems (80 cm capillary, between 8 and 9 min). The injection parameters were optimized and it was shown, that a high sample throughput with a good reproducibility can be achieved.

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Gas chromatography mass spectrometry analysis of saliva samples

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Keywords
GC-MS
saliva
sample pretreatment

Abstract
This work is focused on the development of a new sample pretreatment method for saliva analysis by gas chromatography mass spectrometry techniques (GC-MS, GC-MS/MS). Developed method includes a one-step sample pretreatment and extraction by isopropanol, analytes preconcentration by freeze drying and derivatization with silylation agents hexamethyldisilazane (HMDS) and *N,O*-bis(trimethyl-silyl)trifluoroacetamide (BSTFA). GC-MS/MS analysis of saliva samples was performed in the presence of various metabolites and other substances such as painkiller medicines: ibuprofen and acetaminophen.

1. Introduction

Saliva has hundreds of components that may serve to detect diseases or as evidence of exposure to various substances, as well as provide biomarkers of health. It is a good indicator of plasma levels of various substances, such as hormones or drugs. Healthy adults produce almost 1000 mL of saliva per day. Water is the most represented saliva component, with content up to 99 % of saliva volume. The remaining 1% are complex inorganic (bicarbonate, calcium, fluoride, phosphate, etc.) or organic (enzymes, mucins, immunoglobulines, lipids, proteins, etc.) molecules. Saliva has three major functions: digestion, protection, and lubrication [1]. Saliva can be easily collected from humans. Its compounds are in high concentration levels, because the salivary glands are linked to blood capillaries and there is a straight exchange between blood and saliva. Saliva tests are often used for forensic and clinical purposes or determination of endogenous metabolites [2]. The main advantages of saliva analysis are the easiness and non-invasiveness of sample collection and that it is performable without trained specialist and can be made by the patient or study participant. Unstimulated

whole saliva can be collected with passive drooling or spitting directly into a collector vial or other oral fluid collector and commercial devices [3]. For saliva sample extraction, liquid-liquid extraction is often chosen [4, 5] but micro-extraction techniques are the most common extraction methods [6–8]. Saliva contains a large number of compounds, the structure and function of which have been studied with traditional biochemical techniques, including liquid chromatography, gel electrophoresis, capillary electrophoresis, mass spectrometry, and immunoassays (RIA, IRMA, EIA, ELISA) [3]. Gas chromatography after derivatization of non-volatile compounds is also a suitable method for its analysis [4, 6, 7].

2. Experimental

2.1 Reagents and chemicals

Acetaminophen, *N,O*-bis(tri-methyl-silyl)trifluoroacetamide (BSTFA), and ibuprofen were bought from Sigma-Aldrich. Trifluoroacetic acid was purchased from Fluorochem (UK). Millipore HPLC water, acetonitrile, HMDS were bought from Merck. Isopropanol was purchased from Centralchem (Slovakia).

2.2. Sample pretreatment

Saliva samples were collected to a clean glass container by active spitting during 3 min. After collection, 1 mL of isopropanol was added to 500 µL of sample to a precipitation of salivary mucus and also for the analytes extraction. The solution was thoroughly vortexed, centrifuged (3 min, 5000 RPM), 500 µL of supernatant was collected and diluted with 1 mL of HPLC water. Diluted samples were first frozen at –20 °C and then freeze dried (lyophilized). For the derivatization of non-volatile analytes, the two step derivatization method based on the previously developed method by Podolec et al. [9] with HMDS and BSTFA was chosen. In the first step, 300 µL of HMDS:ACN mixture (1:1, *v/v*) was added as silylation agent to a lyophilized dry extract, 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. In the second step, 200 µL of pure BSTFA was added and the mixture was heated to 80 °C for 30 min. Prepared samples were then directly injected to the gas chromatograph.

2.3 Instrumentation

The gas chromatography mass spectrometry (GC-MS) analyses were carried out with an Agilent 6890 N Network GC System and an Agilent 5973 Network Mass Selective Detector. The injected volume of sample was 1 µL into the injector operating in splitless mode (2 min). The injector temperature was 280 °C. Compounds were separated on a 30 m×0.25 mm (i.d.)×0.25 µm DB-5MS capillary column. The

column temperature was initially set to 50 °C and increased at a rate of 15 °C min^{-1} to 310 °C and was held for 2 min, transfer line temperature was 310 °C. The MS detector with electron impact ionization of 70 eV worked in scan mode in the range of 45–600 amu.

The GC-MS/MS analyses were carried out with a Trace GC Ultra gas chromatograph 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 310 °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 50 °C, held for 2 min and increased at a rate of 15 °C min^{-1} to 270 °C and then increased to 310 °C and was held for 2 min. The specific SRM transitions for analytes were chosen to 160 → 117 at collision energy 20 eV and to 295 → 225 at collision energy 10 eV.

3. Results and discussion

The first step of analysis was the identification of major compounds in saliva samples with GC-MS (Fig. 1). Samples were extracted with isopropanol to remove protein contamination and increase the recovery of analytes extraction. Saliva

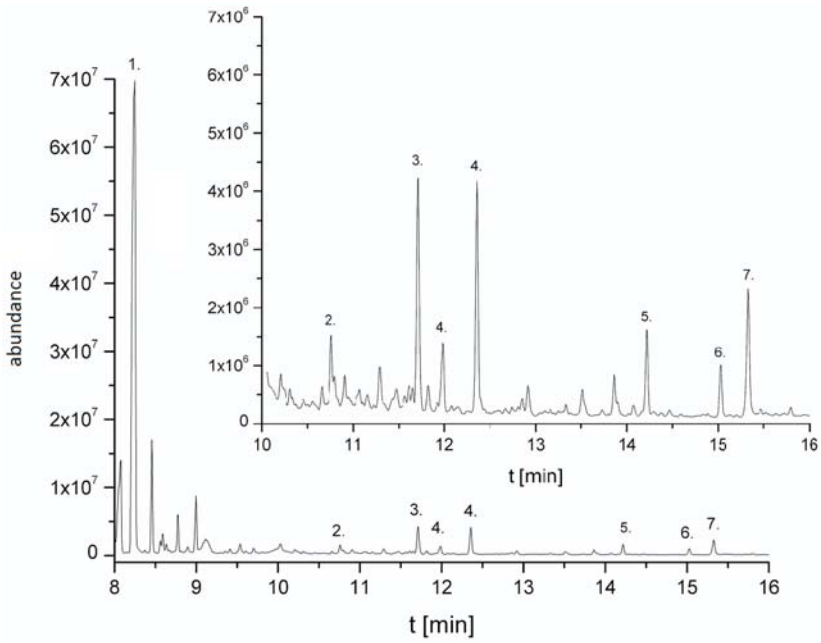

Fig. 1. GC-MS chromatogram of extracted saliva sample in SCAN mode: (1) TMS phosphoric acid, (2) TMS L-oxoproline, (3) TMS GABA, (4) penta TMS glucopyranoside, (5) TMS L-thyrosine, (6) TMS palmitic acid, and (7) tetra TMS uric acid.

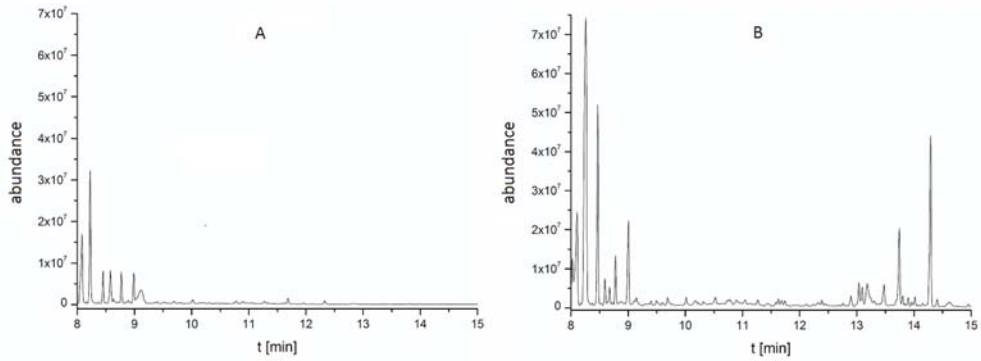

Fig. 2. Comparison of GC-MS chromatograms of saliva samples, (A) before, (B) after meal consumption.

extract contains a large number of analytes. The major compound was trimethylsilyl (TMS) derivative of phosphoric acid. Other major salivary compounds were TMS L-oxoproline, TMS gamma-aminobutyric acid (GABA), penta TMS glucopyranoside, TMS L-thyrosine, TMS palmitic acid and tetra TMS uric acid. For the monitoring of changes in saliva composition, particularly after food intake, we performed a saliva analysis before and after meal consumption. The GC-MS chromatograms of these samples are shown in Fig. 2. The contamination of saliva samples has increased after meal consumption. Because of this, we sampled min. 2 hours after last meal consumption for further analyses. Extraction with isopropanol was selected based on the fact that the pour point after dilution with water was in the range of higher temperatures unlike methanol, which is often used for saliva analytes extraction. This extraction method had a high selectivity and recovery.

The GC-MS/MS standard solution chromatograms of acetaminophen and ibuprofen with a concentration of 50 ppb are shown in Fig. 3. This GC-MS/MS system

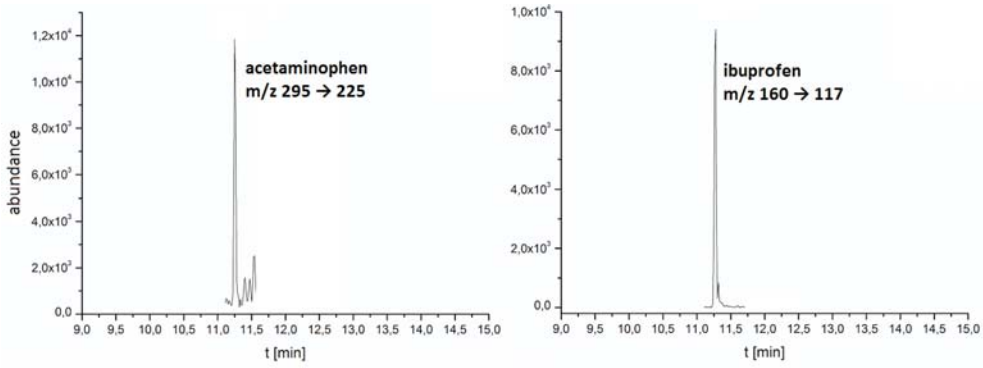

Fig. 3. GC-MS/MS chromatograms of 50 ppb standard solutions of acetaminophen and ibuprofen.

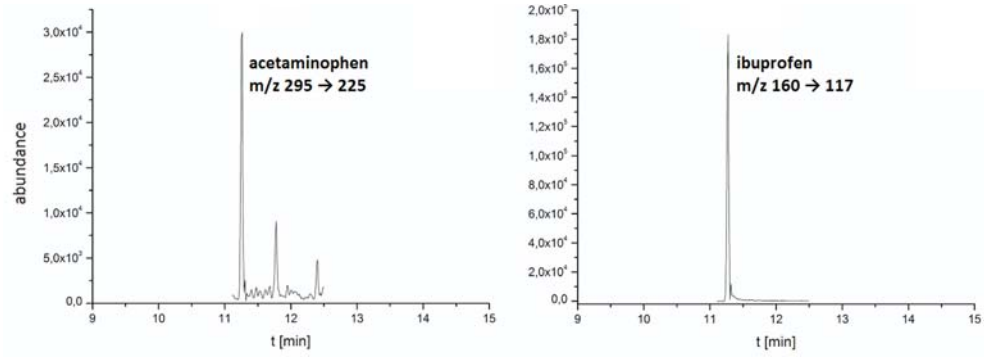

Fig. 4. GC-MS/MS chromatograms of real saliva samples for the presence of acetaminophen and ibuprofen.

allows the analysis of selected compounds of ppb concentration levels and the lower background. Therefore these conditions were applied to real saliva samples from a patient with joint pain. Fig. 4 shows GC-MS/MS chromatograms of real saliva samples. We identified the presence of analgetic and antiphlogistic drugs acetaminophen and ibuprofen, which the patient is regularly taking.

4. Conclusions

This work was focused on the analysis of endogenous end exogenous compounds in saliva by GC-MS and GC-MS/MS techniques. The aim of this study was the development of an extraction method and the preconcentration of analytes by freeze-drying. After preconcentration, derivatization with silylation agents HMDS and BSTFA was performed. This extraction and preconcentration method had a good selectivity and recovery. In real saliva samples we identified a large amount of endogenous metabolites, but also two painkiller drugs which the patient was taking.

Acknowledgments

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Poly(methylene blue) modified carbon based electrodes for the determination of sulfhydryl-containing compounds

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Keywords
carbon-based electrodes
electropolymerization
hydrogen sulfide
poly(methylene blue)
sulfhydryl

Abstract
This research presents the study of electrochemical polymerization of phenothiazine derivative, methylene blue (MB). Electrochemical properties and analytical application of resulting electrocatalytically active poly(methylene blue) (pMB) have been investigated. Three various carbon-based substrates: basal plane of highly oriented pyrolytic graphite (HOPG), pencil graphite electrode (PGE) and carbon fibre micro electrode (CFE) have been used. Aforementioned electrodes modified with the polymer (HOPG/pMB, PGE/pMB, and CFE/pMB) have been prepared by potential cycling in aqueous electrolyte solution containing dissolved monomeric methylene blue. Electrocatalytic activity of three developed electrode systems towards hydrogen sulphide has been compared. Developed electrodes have been further investigated as potential sensors for sulfhydryl-containing compounds.

1. Introduction

In over last decade hydrogen sulfide has been identified as an endogenous signalling molecule of gas [1] with auspicious therapeutic effects [2]. Its endogenous metabolism and physiological functions positioned it as the third member of the novel family of endogenous gaseous transmitters, termed as gasotransmitters, besides nitric oxide and carbon monoxide [3, 4]. Due to very low physiological concentrations and potential therapeutic concentrations, there is growing demand for developing more appropriate analytical methods with emphasis on their sensitivity and selectivity.

Numerous materials including gold, platinum, or carbonaceous materials such as graphite and glassy carbon have been used for sulfhydryl group determination.

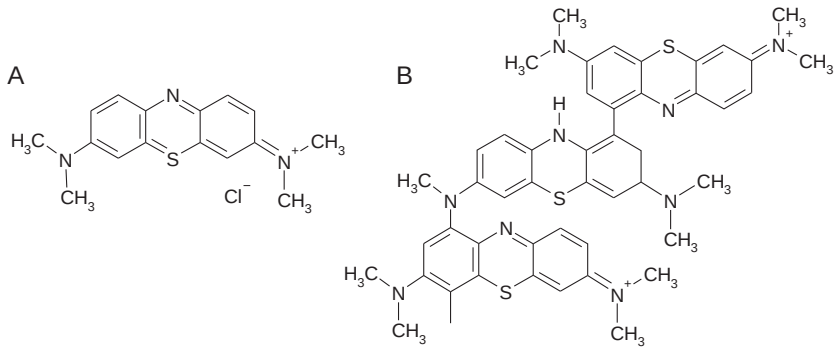

Fig. 1. Chemical structure of (A) methylene blue, and (B) possible structure of poly(methylene blue) as proposed by Giribabu [13].

Carbon-based substrates are extensively employed in electroanalysis with such benefits as chemical inertness, biocompatibility, wide potential range in aqueous solutions, and relatively low cost compared to the precious metal electrode. Using bare electrodes in electroanalysis of sulfhydryl compounds can be complicated by passivation of the electrode surface, impractically high activation overpotentials required for the oxidation or reduction, or even no observed electrochemical oxidation of analytes. However, promising advances towards improved selectivity in carbon-based electrochemical sensors have been achieved through surface modification of the working electrode with redox mediators. Using an appropriate mediator immobilized on the electrode surface can improve the electrochemical response to analyte by facilitating the charge transfer reaction.

In the last two decades, numerous studies focused on surface-modified electrodes created by the electropolymerization of various phenoxazine and phenothiazine-based compounds have been reported [5, 6]. Methylene blue (MB, Fig. 1) is a water-soluble phenothiazine-based cationic dye. Potential cycling in basic aqueous solution with dissolved MB monomer leads to the formation of stable conductive polymer film on the solid electrode surface [7]. This film proved electrocatalytic activity towards some biologically active compounds has been already reported in several studies [8–12].

The aim of our work is designing of new electrode system which combines auspicious electrochemical properties of pMB and advantages of various carbon--based substrates. We optimized the conditions of electropolymerization and characterized the properties of developed modified electrodes. All of them exhibit electrocatalytic activity towards SH^-. Our findings will be utilized for development of new potentiometric sensor.

2. Experimental

2.1 Reagents and chemicals

All of the used chemicals were of analytical grade and were used without further purification. All aqueous solutions were prepared using Millipore Milli-Q pure water (resistivity ≥ 18 MΩ cm). Methylene blue was purchased from Lachema. Aqueous solution used for the electropolymerization consisted of 0.1mM monomeric MB, 0.1M phosphate buffer (pH = 8.0) and 0.1M sodium nitrate. Solution of 0.1M phosphate buffer with 0.1M potassium chloride was used for pMB electrochemical characterization and other experiments. Solution of sodium sulphide was prepared immediately before the experiment by dissolving in supporting electrolyte deaerated by argon gas. All 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 performed with a computer-controlled potentiostat-galvanostat Wenking POS2 (Bank Electronik) and the data were collected using CPC-DA software (Bank Elektronik). The electrochemical experiments were carried out in two types of electrochemical cell depending on used working electrode. All measurements have been done with carbonaceous electrode (bare or modified by pMB) as a working electrode, 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.

2.3 Carbon electrodes design

HOPG substrate was prepared by peeling off basal plane of highly oriented pyrolytic graphite (HOPG, ZYB Grade, 12×12×2 mm; Momentive Performance Materials Quartz) and then was fixed by the double sided tape on the metal wafer. Geometric area accessible for modification was defined by viton flat sealing (20 mm^2). The pencil-graphite rod (HB of 0.5 mm in diameter and 6 cm in length, KOH-I-NOOR) was inserted into a glass capillary and sealed using two-component epoxy adhesive. Electrical contact was obtained by mercury and platinum wire. Carbon fibre (30 μm in diameter) connected to Pt-Ir wire (75:25) by conductive adhesive was inserted into a glass capillary and sealed using UV-cured polymer (Bondic). Unpolymerized residues were then cleaned by immersing in ethanol.

2.4 Electrochemical polymerization

Solution used for the electropolymerization consisted of 0.1mM monomeric MB, 0.1M phosphate buffer (pH = 8.0) and 0.1 M sodium nitrate. The preparation of

pMB films was carried out by potential cycling between –0.6 V and +1.1 V (*vs.* SCE) at the sweep rate 100 mV s^{-1} The polymer growth was controlled by the deposition time or the number of potential cycles. After the electropolymerization both the electrode and the cell were thoroughly rinsed with saturated KCl and phosphate buffer containing 0.1M KCl to remove any remaining monomeric MB.

3. Results and discussion

Fig. 2A represents typical current response during potential cycling in solution of MB monomer between –0.6 V and +1.1 that leads to the polymer film growth on the surface of working electrode. In the potential area positively from 0.8 V the rapidly rising current (wave III) originates from the oxidation of the MB to cation-radicals MB^+ . These radicals are responsible for the methylene blue polymer formation. The decrease in the monomer concentration near the working electrode surface corresponds to a decrease in the oxidation peak at –0.26 V (I, I'). The growth of the polymer film is accompanied by an increase of redox peaks corresponding to the polymeric MB (II, II'). After electropolymerization was performed and cell and electrode rinsed, the integrity of the pMB film modified working electrode was checked by current response on potential cycling in 0.1M phosphate buffer of pH = 8 with 0.1M KCl dissolved, being as in Fig. 3.

Electrocatalytic activity of designed working electrodes was investigated and compared. The current response during potential cycling in presence of 0.01M Na_2S in 0.1M phosphate buffer and 0.1M KCl (pH = 8) was investigated. New anodic peak corresponding to the SH^- oxidation was observed at potential about 0.340 V if the modified electrode has been used. No oxidation peaks

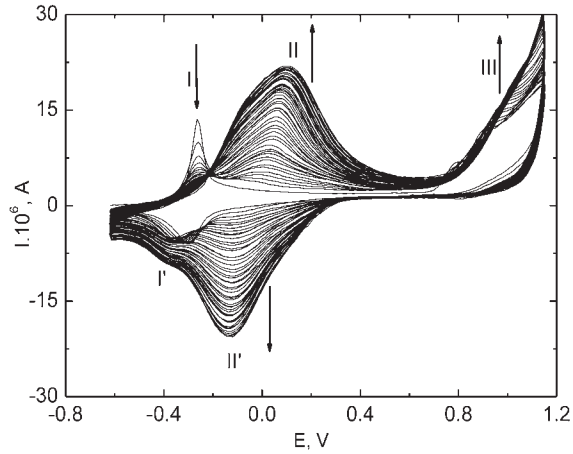

Fig. 2. The pMB film growth during electropolymerization from a solution containing 0.1mM MB monomer in 0.1M phosphate buffer and 0.1M $NaNO_3$ (pH = 8.0) on the surface of HOPG. 30 minutes cycling between –0.6 and +1.1 V *vs.* SCE at scan rate 100 mV s^{-1}.

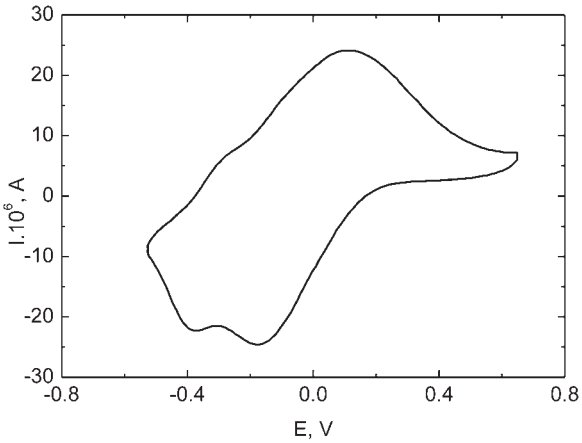

Fig. 3. Typical cyclic voltammogram of pMB modified carbon-based electrode (after 30 min deposition from the 0.1mM mMB solution) in 0.1M phosphate buffer (pH = 8) with 0.1M KCl.

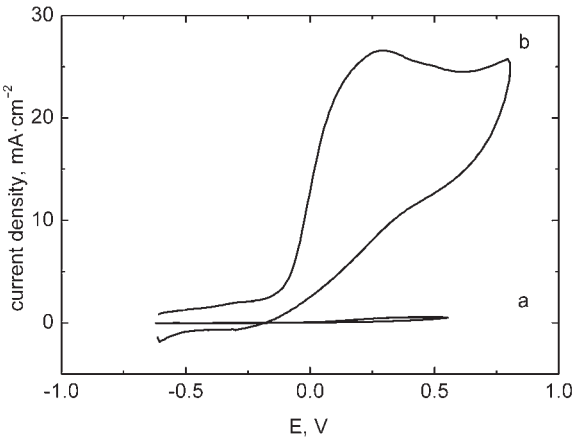

Fig. 4. Comparison of current response (in current density) of (a) HOPG/pMB, and (b) PGE/pMB in 0.1M phosphate buffer with 0.1M KCl (pH = 8.0) containing 0.01M Na_2S.

corresponding to the sulfhydryl oxidation were observed for the bare electrode in defined potential range. It has been found that the electrocatalytic activity strongly depends on the used type of carbon-based electrode (Fig. 4). Based on cyclic voltammetry measurements, we can conclude that pMB film deposited on used substrates displays sufficient electrocatalytic activity towards sulfhydryl group and pMB-modified electrode can thus be utilized for construction of new potentiometric sensors for sulfhydryl-containing compounds.

4. Conclusions

We have investigated and compared electropolymerization process, electrochemical properties and electrocatalytic activity of poly(methylene blue) film modified carbon electrodes. Based on cyclic voltammetry measurements, we can conclude that studied surface modification dramatically improved electrocatalytic activity towards sulfhydryl group. The range of the electrode response strongly depends on the type of used electrode substrate.

Acknowledgments

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Applications of flow-through coulometric detector with renewable working material based on carbon spherical microparticles

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Keywords
coulometric detector
amperometry
HPLC
flow injection analysis

Abstract
This paper maps the development and testing of recently presented flow-through coulometric detector with renewable working material based on spherical carbon microparticles, all the way from its construction and testing to application on pharmaceuticals determination or samples with complex matrix such as urine. It was firstly tested on electrochemically well defined model samples: potassium ferrocyanide and hydroquinone to characterize its general electrochemical properties. Later, the applicability of the detector was proved by determination of tyrosine and thymol by flow injection analysis and of sulfamethizole and mixture containing homovanillic and vanillylmandelic acid by HPLC.

1. Introduction

In modern analytical chemistry, there is great requirement for determination in flow arrangement, especially for flow injection analysis (FIA) and HPLC. One of the key parts of these systems are detectors and so there are also great demands on them, like sensitivity, linearity and mainly reproducibility etc [1]. Electrochemical detectors meet these conditions well and besides, they have an advance in the price of instruments and therefore also the price of an individual analyse. On the other side, the passivation is big disadvantages of electrochemistry, because it can lower the reproducibility.

In flow arrangement, there are two main and the most common electrochemical techniques: amperometry and coulometry. Coulometry works with high conversion degree (almost 100%) and so the working material must have sufficiently large active surface [2, 3]. It can be accomplished by using porous material or material based on microparticles, such as crushed vitreous carbon [4, 5].

Passivation is the most serious problem for these materials, especially in case of determination of samples with complex matrix. The working material must be then periodically and reproducibly renewed. Mechanical cleaning is almost impossible; therefore, the replacement of whole working material is necessary. To overcome this expensive and time-consuming process, special designs of detectors were developed, such as "jet ring" detector, which enables faster and cheaper replacement of passivated working material [6–8].

This work will introduce one such concept from its basic development and testing to analysis of real compound and their determination in model matrix or real samples of pharmaceutical preparations.

2. Experimental

2.1 Chemicals and samples

Stock solutions of potassium ferrocyanide and hydroquinone (Fig. 1) (both Lachema,Czech Republic) of concentration 1×10^{-3}mol L^{-1} were prepared by dissolving the proper amount of the respective compound in distilled water. Thymol (Fig. 1, Tamda, Czech Republic) and sulfamethizole (Fig. 1, 99% Sigma-Aldrich) were dissolved in methanol (≥ 99.9%, Merck, Germany) in the concentrations 1×10^{-2} mol L^{-1} and 1×10^{-3}mol L^{-1}, respectively). Homovanillic acid (HVA, Fig. 1, 97%) and DL-4-hydroxy-3-methoxymandelic acid (VMA, Fig. 1, 98%; both Sigma Aldrich) were dissolved in acetonitrile for HPLC (99,9%, Carlo Erba Reagents, Italy) in the concentration 1×10^{-3} mol L^{-1}. Tyrosine (Fig 1., 1×10^{-3} mol L^{-1}) was

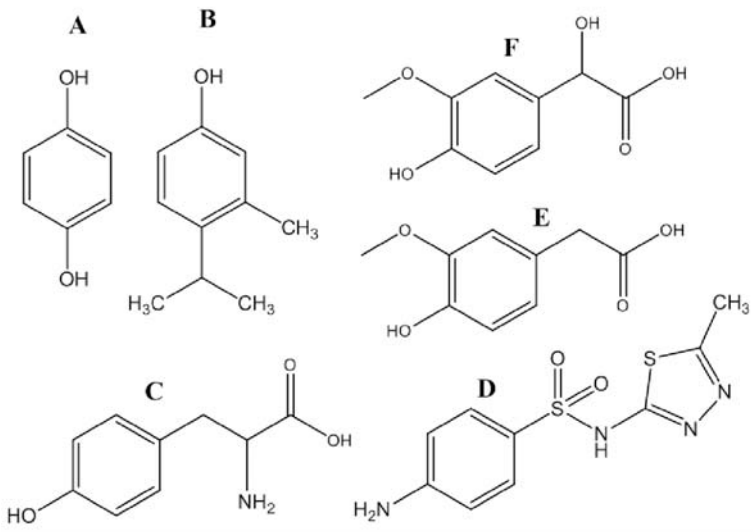

Fig. 1. Chemical structure of (A) hydroquinone, (B) thymol, (C) tyrosine, (D) sulfamethizole, (E) homovanillic acid, and (F) DL-4-hydroxy-3-methoxymandelic acid.

dissolved in solution of 0.1 mol L^{-1} HCl (Lach:Ner, Czech Republic). Solutions of lower concentrations were obtained by dilution of stock solutions by carrier solution or the mobile phase.

Sodium sulfate (Lachema, Czech Republic) of concentration 0.1 mol L^{-1} was used as carrier solution for the measurements with potassium ferrocyanide.

Britton-Robinson (B-R) buffers were used for preparation of carrier solutions and mobile phases. Alkaline component of B-R buffer was prepared from sodium hydroxide (98%, Lach-Ner, Czech Republic) and acidic component from phosphoric acid (85%, Lach-Ner, Czech Republic), boric acid (99.5%, Lach-Ner, Czech Republic), and acetic acid (99%, Lach-Ner, Czech Republic). For the determination of sulfamethizole, HVA and VMA, acidic and alkaline components were ten times diluted by water and then mixed together to obtain proper pH.

For the preparation of mobile phases of HVA and VMA, methanol for HPLC (99,9%, Avantor Performance Materials S.A. de C.V., Mexico) or acetonitrile for HPLC (99,9%, Carlo Erba Reagents, Italy) were used.

Urine samples were ten times diluted by water and the sample was analysed by standard addition method.

Content of thymol and sulfamethizole in tablets Septolete D (Krka, Slovenia) and Micturol® SedanteFuerte (LaboratorioFarmaceutico S.I.T. Srl, Italy) was determined by FIA-ED and HPLC-ED, respectively.

Millipore system Symplica UV (Millipore, USA) was used for the preparation of distilled water for all aqueous solutions.

For the introduction of the working material, 500 μL of suspension consisting of 10 mg of spherical microparticles of glassy carbon (size 10–20 μm, Alfa Aesar, Germany) and 3 mL of nitromethane (POCH, Poland) were injected to the detector; nitromethane was washed away by flow of methanol.

2.2 Instrumentation

High pressure pump HPP 4001 (Laboratorni pristroje Praha, Czech Republic) with 6-port injection valve equipped with loop of 0.75 mm inner diameter (Rheodyne, USA) were used for the filling.

HPLC system for HVA and VMA determination was consisted of HPLC pump LC-6A Liquid chomatograph, SPD-6A UV spectrophotometric detector, CBM-10A communications bus module (all Shimadzu, Japan), PalmSens Electrochemical Sensor Interface for electrochemical detection (PalmSens, Netherlands), column KromasilEthernity 5-Phenylhexyl (AkzoNobel, USA) and 6-port valve (Rheodyne, USA). Injected volume was set for 20 μL. UV detector was set on 247 nm.

FIA apparatus consisted of high pressure pump HPP 5001, ADLC2 detector (both Laboratorni pristroje Praha, Czech Republic) and 6-port injection valve (Rheodyne, USA).

HPLC system for sulfamethizole determination consisted of the same pump, detector, and injection valve as in the case of FIA. Column Lichrospher® RP-18,

Table 1
Optimal carrier solutions or mobile phases and determination method.

Sample	Technique	Carrier solution/mobile phase
$K_4[Fe(CN)_6]$	FIA-ED	0.1 mol L^{-1} sodium sulfate
hydroquinone	FIA-ED	B-R buffer of pH = 2 [9]
thymol	FIA-ED/UV	B-R buffer of pH = 10
sulfamethizole	HPLC-ED/UV, linear pump	methanol and 10× diluted B-R buffer of pH = 3 (70:30, *v/v*) [10]
tyrosine	FIA-ED	B-R buffer of pH = 4.5
HVA and VMA	HPLC-ED/UV, gradient pump	10× diluted B-R buffer of pH = 3 and acetonitrile 75:25 (*v/v*)

100 (5 μm), 125×4 mm (LichroCART, Merck, Germany) was used for the separation. Spectrophotometric detector Sapphire (ECOM, Czech Republic) was set on 275 nm for determination of thymol at 277 nm for the determination of sulfamethizole.

For all the measurements, working coulometric electrode was set in 3-electrode arrangement with platinum sheet auxiliary electrode and Ag/AgCl (3 M KCl) reference electrode (both Monokrystaly Turnov, Czech Republic).

Optimal carrier solutions or mobile phases and determination methods are tablutated in Table 1.

2.3 Procedures

Concentration dependences were evaluated by least squares linear regression method. Limits of quantification were calculated as ten times the standard derivation ($\alpha = 0.05$), calculated from ten repeated measurements of the lowest concentration of the determined analyte, divided by the slope of calibration dependence [11]. All the measurements were made in triplicate, unless stated otherwise.

Content of thymol and sulfamethizole in pharmaceuticals and of HVA and VMA in urine samples was determined by newly developed detector and by standard method (HPLC with spectrophotometric detection). For determination of HVA and VMA in human urine standard addition method was employed.

3. Results and discussion

3.1 Development

The scheme of detector can be seen in the Fig. 2 together with its part and material description. Before the final detector version, different construction procedures and other construction materials were tested. Coulometric detector is based on thermally flanged Teflon capillary (inner diameter 0.5 mm). PEEK capillaries,

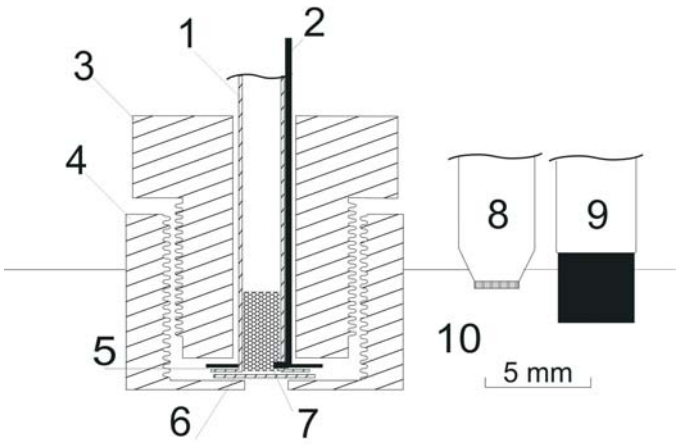

Fig. 2. Schematic representation of coulometric detector: (1) Teflon capillary (diameter 0.5 mm), (2) platinum wire, (3) screw, (4) cap, (5) iron ring, (6) filtration paper, (7) working material (glassy carbon microbeads), (8) reference and (9) auxiliary electrode in (10) mobile phase or carrier solution.

which would provide better pressure resistance, do not enable thermal flanging, which is used for fixation of capillary inside of the detector. A screw and a cap with a hole are used for holding a filter made of filtration paper on the outlet of the capillary. Near the flanged end, platinum wire of diameter 0.4 serving as the electrical contact was introduced through the capillary wall and isolated by a polymer (polystyrene) and heat-shrink tubing. Contact wire of diameter 0.1 mm was also tested, but it was too soft and also the disproportion between the diameter of the wire and the diameter of needle used for the puncture of the capillary led to leaks of mobile phases from the detector through the capillary wall.

3.2 Handling

Working material was filled into the detector by flow of methanol (1 ml min^{-1}); Fig. 3. This procedure results in the deposition of column of carbon microparticles with the height about 4 mm. Working material was changed according to the requirement of the determination, usually due to a passivation, by dismounting the filter and washing out the spent working material by methanol.

3.3 Testing of the detector using hydroquinone and ferrocyanide as model compounds

Basic electrochemical parameters were tested using ferrocyanide and hydroquinone as model compounds. Main parameter of coulometric detectors is the conversion degree. It was determined from area of peaks measured under the optimal condition (Table 2) and for both compounds were around 100% (99.8%

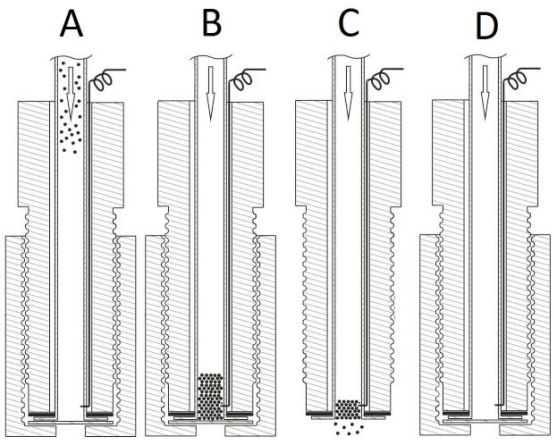

Fig. 3. Filling of the detector: (A) beads are introduced into the detector by flow of methanol, (B) beads are captured inside of the detector and samples can be injected, (C) filter is removed and working material is washed out by flow of methanol, (D) filter and cap are back on their places and detector is ready for refilling.

Table 2
The optimal conditions of determination.

Sample	Detection potential [V]	Flow rate [mL min^{-1}]	Injected volume [μL]
$K_4[Fe(CN)_6]$	+0.6	0.7	100
hydroquinone	+1.1	0.7	100
thymol	+1.1	0.6	50
sulfamethizole	+1.6	0.8	20
tyrosine	+1.3	0.6	50
HVA and VMA	+1.3	0.7	20

for ferrocyanide and 109.0% for hydroquinone). As was mentioned in the introduction, modern analysis demands high reproducibility of measurements. Therefore, the robustness of the detector response was tested by calculating of relative standard deviation (RSD) of signal for five different working material filled in the detector. For both model compounds, RSD were around 2%. The length of working material inside of the detector reaches approx. 4 mm. Similar value was also calculated from the performance parameters of the detector [12, 13] and so it can be supposed that whole working material was active during determination.

Linearity of the signal was tested by measuring the calibration curves for both model compounds (Fig. 4). Calibration curves were linear from 8 to 100 μmol L^{-1} for ferrocyanide and from 6 to 100μmol L^{-1} for hydroquinone.

For a reversible oxidation, hydrodynamic voltammograms can by theoretically described [14]. Hydrodynamic voltammograms were measured and compared

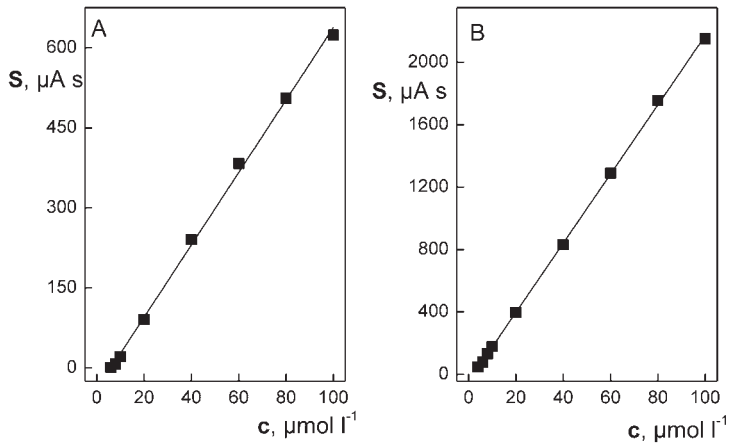

Fig. 4. Calibration dependences of (A) ferrocyanide, and (B) hydroquinone, measured under the optimal conditions.

with theory. The slopes of rising part of ferrocyanide hydrodynamic voltammogram were lower than theoretically expected, but for hydroquinone, the slope was in good agreement with theory.

3.4 Application of the detector

Basic electrochemical properties were good enough for application of the detector for determination of real and more complex samples. For this purpose, different type of analytes were chosen: thymol, tyrosine, sulfamethizole, HVA, and VMA.

3.5 FIA determination of thymol and tyrosine:

Tyrosine is one of the 22 amino acids that are used by cells to synthesize proteins. The FIA was used for determination of this significant compound. For tyrosine, optimal conditions of determination were found using multivariate statistical method – fractional factorial design. Under the optimal conditions, calibration curve was measured from 1×10^{-4} to 2×10^{-6} mol L^{-1} and detection limit was calculated. Optimal conditions and parameters of the calibration curve are shown in Table 2 and 3, respectively.

Another organic compound analysed by FIA was thymol (Fig. 5). Thymol was chosen because it has high passivation abilities, especially towards glassy carbon electrode material. Thymol has antibacterial and antiseptic properties [15] it is component of many herbs and suppressors of growth of bacteria, viruses and fungi. Thymol is therefore used for therapy of diseases of respiratory tract [16] and as an antisepticum in stomatology. Optimal conditions for determination of thymol, namely pH of B-R buffer, flow rate, detection potential and injected volume were found and are demonstrated in Table 2.

Table 3
Figures of merits of calibration dependences.

Sample	slope [μA s μmol^{-1}L]	intercept [μA s]	correlation coefficient	*LOQ* [μmol L^{-1}]
$K_4[Fe(CN)_6]$	6.79	40.8	0.9981	1.25
hydroquinone	22.18	82.2	0.9997	1.64
thymol	7.65	18.3	0.9991	0.97
sulfamethizole	3.59	1.1	0.9987	0.04
tyrosine	10.66	5.37	0.9909	2.11
HVA	2.12	0.61	0.9977	0.15
VMA	2.55	40.0	0.9956	0.27

Table 4
Robustness of signal.

Sample	RSD on one filling	Number of stable injection	RSD of five consecutive fillings
$K_4[Fe(CN)_6]$	1.8	30	4.9
hydroquinone	2.1	20	4.4
thymol	1.6	6	4.2
sulfamethizole	2:9	3	6.3
tyrosine	2.7	3	5.0
HVA and VMA	under 4	10	around 4

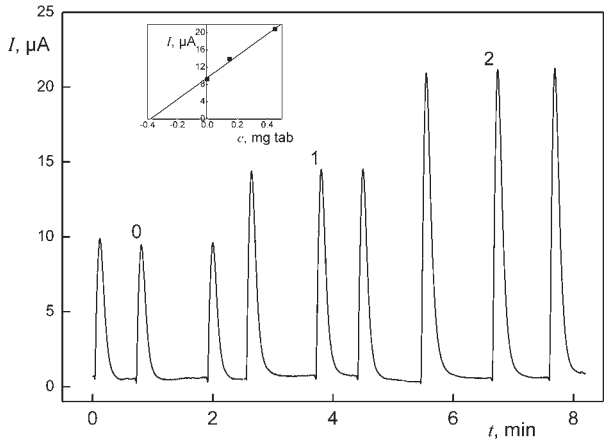

Fig. 5. Sample of pharmaceuticals containing thymol (determination of thymol in tablet Septolete D), standard additions of: (0) 0, (1) 20, and (2) 40 of thymol stock solution (inset shows corresponding concentration dependence). Carrier solution B-R buffer pH = 10, E_{det} = +1.1 V, flow rate 0.6 mL min^{-1} and injected volume 50 μL

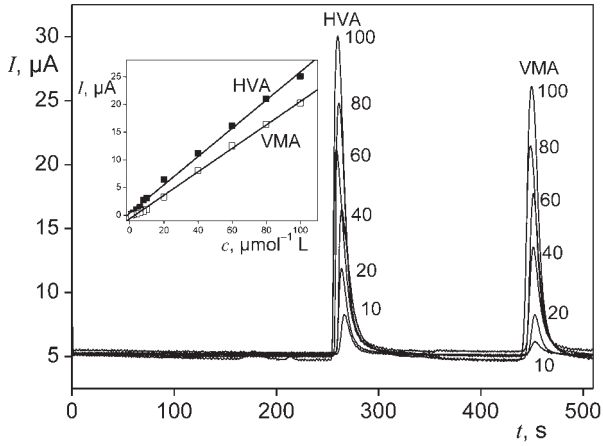

Fig. 6. Calibration dependences of HVA and VMA measured under the optimal condition without gradient program.

Gradual passivation of working electrode material during measurements was observed. The signal of first six injections was stable with RSD 1.6%, but 35% drop of the peak area was observed during next four measurements. Therefore, working material was changed after six sample injections.

Calibration dependence of thymol was measured under the optimal conditions and its parameters are presented in Table 3. Thymol concentration in pharmaceutical preparation Septolete D was determined by standard addition method using the above found optimal conditions (Table 5).

3.6 HPLC determination of sulfamethizole and HVA and VMA

Consecutive step in testing of the detector was application for HPLC (Fig. 6), which is much more common method in analytical laboratories than FIA. The first

Table 5
Summary of real and model samples. Content of thymol and sulfamethizole in tablets declared, determined by the newly developed methods, and determined by HPLC-UV; uncertainty presented by confidence limit (α = 0.05).

Compound	Sample	Content [mg/tablet]		
		Declared	Developed method	HPLC-UV
thymol	Septolete D	–[a]	0.40±0.04	0.39±0.04
sulfamethizole	Micturol® Sedante	250	252.6±18.6	249.9±8.1

[a] Not declared by the manufacturer.

experiments were performed with sulfamethizole, sulfamide antibiotic used mainly against gram-positive and gram-negative bacteria [17, 18]. For determination of sulfamethizole, linear pump was used, because the baseline is more stable in this arrangement. Optimal conditions of its determination were found (Table 2) and calibration dependence was measured under the optimal conditions (Table 3). After verification of linearity of responds, samples of pharmaceuticals were measured by standard addition method (Table 5).

The last step of the detector testing was HPLC with gradient program. Determination of HVA and VMA, final products of catecholamine metabolism, which can serve as neuron transmitters or hormones [19] was used for the purpose. The optimal conditions of HVA and VMA determination were found using isocratic system and are shown in Table 2. Linearity of response was tested for both compounds measuring the calibration curves under the optimal conditions. Under these conditions gradient method for separation of HMA and VMA from urine matrix were developed and human urine sample was measured.

4. Conclusion

In this paper, results measured by flow-through coulometric detector with renewable working material based on glassy carbon spherical microparticles were summarized. Its basic electrochemical parameters were described and possible application on different types of organic and anorganic samples were presented. Detector was tested for two most common analytical methods in flow arrangement FIA and HPLC with growing analytical instrumentation severity: from FIA with linear pump and pure substance determination until HPLC with gradient program for complex sample matrix. Based on presented results, the detector can be used for determination of electrochemically oxidizable substances with benefit of easy renewing of passivated material.

Acknowledgments

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Voltammetric determination of pyruvic acid after its derivatization with *o*-phenylenediamine

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Keywords
differential pulse voltammetry
direct current voltammetry
mercury meniscus modified silver solid amalgam electrode
o-phenylenediamine
pyruvic acid

Abstract
The aim of this contribution was to develop sensitive, inexpensive, and less time-consuming voltammetric methods for the determination of pyruvic acid based on its derivatization with *o*-phenylenediamine, the resulting reaction product being determined by direct current voltammetry and differential pulse voltammetry at a mercury meniscus modified silver solid amalgam electrode. The optimization consisted of finding a suitable medium and regeneration of the electrode surface for the determination of 2-hydroxy-3-methylquinoxaline, a product of a derivatization reaction, and optimization of conditions for the derivatization reaction. The optimal medium was a solution of the Britton-Robinson buffer of pH = 4.0 and the optimal regeneration potentials were E_{in} = –500 mV and E_{fin} = –900 mV for both techniques. Optimization of the derivatization reaction consisted of finding the optimal concentration of *o*-phenylenediamine (2.0×10^{-3} mol L^{-1}), pH of the reaction (1.2), reaction time (30 min), and reaction temperature (35 °C).

1. Introduction

Pyruvic acid is an important substance in fermentative metabolism of sugar in yeast like *Saccharomyces cerevisiae* (main type of yeast in the fermentation of beer or wine) [1]. In an anaerobic medium, it is not possible to use a respiratory chain, so pyruvate is changed by the enzyme pyruvate decarboxylase to acetaldehyde and, in the next step, by alcohol dehydrogenase to ethanol [2]. Concentration of the acid correlates with the maximum growth of the microorganisms when the highest concentration is just before the maximum growth and subsequently the acid is converted to acetate [3]. The final concentration depends on the separation of yeast. This knowledge can be used for the timing of the end of fermentation for alcoholic beverages [4].

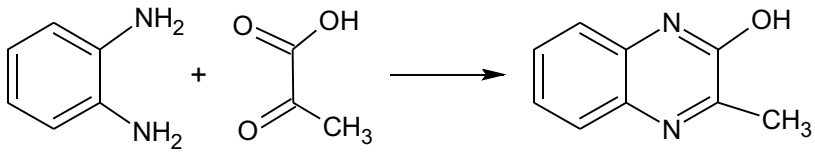

Fig. 1. Reaction of *o*-phenylendiamine with pyruvic acid giving 2-hydroxy-3-methylquinoxaline.

One of the possibilities for determination of pyruvic acid is its derivatization by *o*-phenylenediamine because of its specific reaction with substances containing vicinal diketone group [5]. Aromatic quinoxaline ring is formed during the intermolecular condensation [6], the resultant product being polarographically active [7]. Reaction scheme is in Fig. 1.

Mercury is the best material for polarographic and voltammetric measurements, but due to its toxicity, alternative materials are being sought. Amalgam electrodes were developed in an attempt to replace metallic mercury for a non-toxic alternative material – dental amalgam [8].

2. Experimental

2.1 Reagents

Solutions of 2-hydroxy-3-methylquinoxalin (2-OH-3-MQ; Sigma-Aldrich, purity AldrichCPR; 1×10^{-3} mol L^{-1}) and pyruvic acid (Sigma-Aldrich, 98%; 1×10^{-2} mol L^{-1}) were prepared by dissolving in deionized water (Milli-Q Plus system, Millipore). Solutions of various concentrations of *o*-phenylenediamine (OPDA; Sigma-Aldrich, 99.5%) were stored at a dark place in a fridge and prepared daily due to their limited stability. For the study of the influence of pH on the derivatization reaction, 1 mol L^{-1} hydrochloric acid (HCl; Lach-Ner, p.a., 35%) was used. The Britton-Robinson buffers (BR-buffer) of concentration 0.04 mol L^{-1} was prepared in a usual way. A solution of potassium chloride (KCl; Lach-Ner, p.a.; 0.2 mol L^{-1}) in deionized water was prepared for the activation of the working electrode.

2.2 Instrumentation

All determinations were performed using Eco-Tribo Polarograph with software Polar Pro 5.1 (Polaro-sensors) in a three-electrode arrangement. A mercury meniscus modified silver solid amalgam electrode (m-AgSAE, ETP CZ O X0401) was used as a working electrode, an argentochloride electrode (3 mol L^{-1} KCl, type RAE 113, Monokrystaly Turnov) was used as a reference electrode, and a platinum wire electrode was used as an auxiliary electrode. The scan rate used for both direct current voltammetry (DCV) and differential pulse voltammetry (DPV) was 20 mV s^{-1}. For DPV a pulse height was –50 mV and a pulse width was 100 ms.

A major problem of any solid electrode is the passivation of the electrode surface. For this reason, following cleaning steps were performed [8]: (i) amalgamation (performed once a week or if passivation of the electrode was obvious; a polished silver solid amalgam electrode was dipped in liquid mercury for 20 s and a new meniscus was formed), (ii) activation (the m-AgSAE was polarized in a three-electrode arrangement with a voltage of –2200 mV for 5 min in a stirred solution of 0.2 mol L^{-1} KCl; thanks to this procedure, the passivating compounds absorbed on the electrode surface were removed; activation was performed after amalgamation, at least once a day or in the case of strong passivation, before starting each measurement), and (iii) regeneration (the m-AgSAE was alternately polarized with regeneration potentials a more positive regeneration potential (E_{in}) and a more negative regeneration potential (E_{fin}); pulse cycles were applied for 30 s (150 cycles) and one pulse length was 0.1 s; values of the regeneration potentials were found experimentally by a trial and error method).

The precise pH value was measured using a digital laboratory pH meter Jenway 3510 with a combined glass electrode (type 924 005, Jenway). For tempering solutions for the derivatization reaction, an ultrasonic water bath Fisher Brand (type FB15061) was used.

2.3 Voltammetric measurements

Measurements of the influence of pH and repeatability measurements were performed in solutions prepared by pipetting 1 mL of stock solution of 2-OH-3-MQ (1×10^{-3} mol L^{-1}) to a 10 mL volumetric flask and made up to the mark with the appropriate BR-buffer. Repeatability of individual determinations was measured by carrying out twenty consecutive determinations in the BR-buffer of the optimal pH. Under optimal conditions, when the substance gave the highest response, calibration curves were measured in the concentration range from 1×10^{-6} mol L^{-1} to 1×10^{-4} mol L^{-1}. Before each measurement, the solution was bubbled with nitrogen (Linde, purity 4.0) for 5 min. To investigate the influence of pH (2.0–13.0), voltammograms were measured five times and for repeatability measurements twenty times. For concentration dependences, voltammograms were measured five times and the lowest concentration was measured ten times.

The efficiency of the derivatization reactions at various OPDA concentrations, reaction pH, reaction time, and reaction temperature, were investigated in solutions prepared by pipetting 10 μL of pyruvic acid (1×10^{-2} mol L^{-1}) and 1 mL of the OPDA solution into 10 mL volumetric flasks and filling up to the mark by the BR-buffer. Solutions of OPDA ($0.1–2.0\times10^{-3}$ mol L^{-1}) were prepared by its dissolving in deionized water with added HCl; volume of the acid was calculated to lower the solution pH to values 0.2–2.2. The total reaction time was the time from adding the reagent and completion of the flask to the mark by the appropriate BR-buffer. For comparison of the efficiency of the reaction, the solution of 2-OH-3-MQ standard was used. This solution was prepared by pipetting 1 mL of the standard

stock solution and 1 mL of HCl (concentration of the acid corresponded to pH range from 0.2 to 2.2,) in 10 mL volumetric flasks, made up to the mark with the BR-buffer to pH 4.0. In the investigation of the efficiency of derivatization reactions all measurements were made five times.

To create graphs and to preform calculations and statistics (all at the significance level of α = 0.05), Origin Pro 8.0 (OriginLab Corporation) and Microsoft Excel 2013 (Microsoft Corporation) were used. The limit of quantification (LOQ) was calculated as the concentration equivalent to ten times the standard deviation of ten consecutive measurements of the lowest concentration of the relevant calibration curve [9].

3. Results and discussion

3.1 Voltammetric determination of the product of the derivatization reactions

The dependence of the electrochemical behavior of 2-OH-3-MQ on the pH was studied using DCV and DPV in the BR-buffers in the pH range from 2.0 to 13.0. For both techniques, 2-OH-3-MQ (1×10^{-4} mol L^{-1}) provided one major peak at all pHs and the potential of this peak was linearly shifted to negative values with increasing pH. Maximal response for both techniques was obtained at pH 4.0. Repeatability was measured by twenty consecutive DCV and DPV determinations of 1×10^{-4} mol L^{-1} 2-OH-3-MQ at pH 4.0. The regeneration potentials E_{in} = −500 mV and E_{fin} = −900 mV were chosen for both techniques as the optimal ones for the lowest relative standard deviation obtain. The dependences of the peak height on the concentration were measured under the optimal conditions found. The parameters of calibrations the straight lines and the limits of quantification for both techniques are summarized in Table 1.

Table 1
Parameters of calibration straight lines for DCV and DPV determinations of 2-OH-3-MQ at the m-AgSAE in the BR-buffer pH = 4.0, with applied regeneration potentials E_{in} = −500 mV and E_{fin} = −900 mV (r^2 is coefficient of determination, *LOQ* is limit of quantification (n = 10; α = 0.05)).

Concentration range [mol L^{-1}]	Technique	Slope×10^6 [nA L mol L^{-1}]	Intercept [nA]	r^2	*LOQ* [mol L^{-1}]
$(1–10)\times10^{-5}$	DCV	−0.960±0.006	−0.90±0.34	0,9998	–
	DPV	−1.112±0.008	−0.37±0.51	0.9997	–
$(1–10)\times10^{-6}$	DCV	−1.021±0.006	−0.06±0.04	0.9998	4.8×10^{-7}
	DPV	−1.108±0.004	−0.14±0.03	1.0000	3.4×10^{-7}

3.2 Derivatization reaction

For study of the derivatization reaction, DPV was chosen because of its lower limit of quantification. Study of the influence of the concentration of OPDA ($0.1–2.0\times10^{-3}$ mol L^{-1}; pyruvic acid concentration of 1×10^{-4} mol L^{-1}) on the derivatization reaction was carried out at pH 1.6 and at the reaction time of 30 min (results from preliminary experiment). The optimal concentration of OPDA was 2.0×10^{-3} mol L^{-1}. The dependence of the derivatization reaction on the pH was examined within the pH range from 0.2 to 2.2. The highest efficiency was observed at pH 1.2. The influence of the reaction time and the reaction temperature was measured in several different versions. Despite the fact that a longer time would be optimal, the reaction time of 30 min with the reaction temperature of 35 °C were chosen. Repeatability of the derivatization reaction yield was evaluated from ten independent determinations of pyruvic acid and this repeatability was found to be 3.1% (conditions: $c_{(OPDA)} = 2.0\times10^{-3}$ mol L^{-1}, reaction time of 30 min, reaction temperature of 35 °C).

4. Conclusions

In this work, optimal conditions were found for the determination of pyruvic acid after its derivatization with o-phenylendiamine (OPDA) (subsequent determination of the reaction product, 2-hydroxy-3-methylquinoxalin) by direct current voltammetry (DCV) and differential pulse voltammetry (DPV) at a mercury meniscus modified silver solid amalgam electrode (m-AgSAE). The optimal conditions for the determination were: Britton-Robinson buffer of pH 4.0 and regeneration potentials E_{in} = –500 mV and E_{fin} = –900 mV for both techniques. The calibration curves were measured under the optimal conditions in the concentration range of $1\times10^{-6}–1\times10^{-4}$ mol L^{-1}. The limit of quantification (*LOQ*) for DCV at the m-AgSAE was 4.8×10^{-7} mol L^{-1} and for DPV at the m-AgSAE it was 3.4×10^{-7} mol L^{-1}. The optimal conditions for the derivatization reaction were: concentration of OPDA of 2.0×10^{-3} mol L^{-1}, pH of the reaction of 1.2, time of the reaction of 30 min, and temperature of the reaction of 35 °C.

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Voltammetric determination of homovanillic acid at boron doped diamond electrode for hollow fibre microextraction

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Abstract
Presented work deals with hollow-fibre assisted liquid/liquid/liquid microextraction (HF-LPME) and subsequent voltammetric detection of clinical biomarker homovanillic acid (HVA). Three main areas are discussed: hollow fibre assisted extraction of HVA, voltammetric detection of HVA under basic conditions, and sensing in very small volumes. Using HF-PLME, HVA was extracted with 1-octanol as a supported liquid membrane with enrichment factor of 55 after 60 minutes of extraction. A novel method of miniaturized voltammetric determination of HVA using cathodically pretreated boron doped diamond electrode was also developed. Using differential pulse voltammetry, limit of detection was 1.9 μmol dm^{-3}, limit of quantification 6.6 μmol dm^{-3}.

1. Introduction

Homovanillic acid (3-methoxy-4-hydroxyphenyl acetic acid, HVA) is a final stage metabolite of dopamine and important clinical biomarker [1]. HVA concentration is used as a marker of various oncological and neurological disorders. For example, malign tumors of adrenal glands, pheochromocytoma and neuroblastoma are linked with elevated levels of HVA [2]. For clinical purposes, urinary concentration of HVA is often determined alongside vanillylmandelic acid and creatinine. Fast, sensitive and inexpensive determination of HVA in biological fluids, especially urine, is crucial for possible large scale screenings for diseases.

For this purpose, combination of hollow fibre liquid phase microextraction (HF-LPME) with electrochemical methods is very promising. HF-LPME is a relatively new method for biological sample preparation [3]. The hollow fibres are typically made from thin porous polypropylene (pore diameter 0.2 μm). The fibre

serves either as a separator between phases (e.g. water/1-octanol) in two phase extraction, or more commonly, as a carrier of a water immiscible liquid membrane. Utilization of liquid membrane in a three-phase system features low consumption of organic phase and possible extraction of ionisable species into aqueous phase. Acidic species are extracted from acidic solution through water immiscible liquid membrane to the basic solution inside the fibre. The aqueous extract is also convenient for subsequent voltammetric detection.

2. Experimental

2.1 Reagents and chemicals

The stock solution of HVA (1×10^{-3} mol dm^{-3}) was prepared by dissolving of 18.2 mg of solid substance 4-hydroxy-3-methoxyphenylacetic acid (fluorometric reagent, Sigma-Aldrich) in 100 cm^{-3} of deionized water using ultrasonic bath. It was stored in dark and cold. All other chemicals used were at least of analytical grade purity. For all the measurements, deionized water from Milli-Q-Gradient, Millipore, Prague, Czech Republic (conductivity < 0.05 μS cm^{-1}) was used.

2.2 Instrumentation

Voltammetric measurements were executed with the computer controlled Eco--Tribo Polarograph (Polaro-Sensors, Czech Republic), equipped by MultiElChem 3.1 software for Windows XP/7/8 (J. Heyrovský Institute of Physical Chemistry of the AS CR, v.v.i., Czech Republic). Miniaturized three electrode system was used with working boron doped diamond electrode (BDDE, geometric area with geometric area of 16.5 mm^2), platinum wire counter electrode and reference Ag/AgCl/saturated KCl electrode in Luggin capillary. Luggin capillary was filled with 0.1M KOH. Working BDDE was prepared by microwave assisted plasma CVD with 4000 ppm of B_2H_6 in feed gas.

2.3 Procedures

Prior to each measurement, BDDE was cathodically pretreated by applying potential of −1500 mV for 15 s. DPV was carried out at a scan rate of 20 mV s^{-1}, the pulse amplitude of 50 mV, pulse duration of 100 ms, sampling time of 20 ms beginning 80 ms after the onset of the pulse and interval between pulses of 100 ms. For HF-LPME, 10 cm of hollow fiber was rinsed in acetone, dried in the flow of nitrogen and immersed into 1-octanol for 10 seconds to fill the fibre pores. Afterwards, the hollow fibre was sonicated for 30 seconds at the ultrasonic bath to remove residuals of 1-octanol. Hollow fiber filled with 0.1 KOH was immersed into stirred 10 ml solution of 10^{-5} mol dm^{-3} HVA acidified with 1 ml of 0.1 mol dm^{-3} HCl. After 60 minutes, 12 μL of acceptor solution was transferred by Hammilton syringe directly on the BDDE surface for voltammetric analysis.

3. Results and discussion

For determination of HVA in the single 12 μL drop of hollow fiber extract, home-made miniaturized three electrode system was used with upside-down arranged working electrode. After extraction, the extract was applied on the surface of the working electrode. Platinum wire sealed in glass micropipette and Luggin capillary with Ag/AgCl/KCl(saturated) reference electrode were immersed into the drop. Alternatively pseudo-reference electrodes, such as platinum black or iridium/iridium oxide electrodes could be also used, either in three or two electrode system.

Since majority of reported voltammetric methods for determination of HVA uses of acidic conditions of pH 2 or 3 [4, 5], a new procedure for determination in inherently basic hollow fiber extract was developed. Best performance was obtained with the cathodically pretrated boron doped diamond electrode. Pretreatment of the electrode is of the utmost importance. For determination of HVA under acidic conditions, in which HVA molecule is not charged, anodic pretreatment (application of +2400 mV for 15 seconds prior to the measurement) provides the lowest background and highest and most stable peaks. However, in the case of negatively charged HVA, which is present under basic conditions, only a poorly developed peak could be recorded in the area of electrolyte decomposition around 1.3 V. This effect can be explained by electrostatic repulsion between negatively charged oxygen-terminated BDDE surface and negatively charged HVA molecules. On the other hand, when the BDDE was pretreated cathodically by applying potential of –2400 mV for 15 seconds, background gradually rose, overlapping the HVA peak. Repeatability was rather poor, observable peak height dropped by 40% after five consecutive measurements. Optimum conditions of BDDE pretreatment were found experimentally, the optimum potential was –1500 mV, which was applied for 15 seconds before each measurement. The repeatability of the procedure was assessed on the basis of ten consecutive DPV measurements of 100μM HVA in 0.1M KOH. Calculated RSD was 1.22%. One, well developed oxidation peak of HVA was observed at 420 mV (Fig. 1 and 2). Concentration dependencies of HVA were linear, as is apparent in equations
a) for HVA in the range 0–10 μM

$$I_p\,[\mathrm{nA}] = (10.96 \pm 0.75)\, c\,[10^{-6}\,\mathrm{mol\,dm^{-3}}] + 77 \pm 47 \qquad (1)$$
$$R^2 = 0.997$$

b) for HVA in the range 0–100 μM

$$I_p\,[\mathrm{nA}] = (15.70 \pm 0.58)\, c\,[10^{-6}\,\mathrm{mol\,dm^{-3}}] + 8.0 \pm 0.58 \qquad (2)$$
$$R^2 = 0.996$$

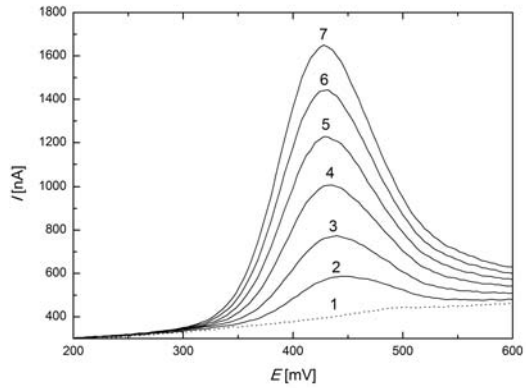

Fig. 1. DPV curves of 0–100μM HVA at BDDE in 0.1M KOH. HVA concentrations of 0 (1), 10 (2), 20 (3), 40 (4), 60 (5), 80 (6), and 100 (7) μmol dm^{-3}.

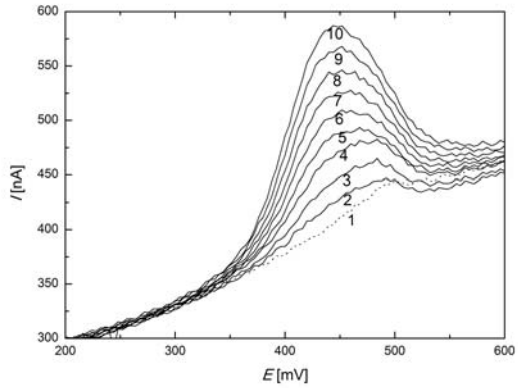

Fig. 2. DPV curves of 0–10μM HVA at BDDE in 0.1M KOH. HVA concentrations of 0 (1), 2 (2), 3 (3), 4 (4), 5 (5), 6 (6), 7 (7), 8 (8), 9(9), and 10 (10) μmol dm^{-3}.

The optimized method was used for pilot experiment with hollow fibre micro-extraction of HVA. 1-octanol was used as the liquid membrane supported by the fiber. After 60 minutes of extraction from acidified 10 μM solution of HVA into 0.1M KOH acceptor solution. Obtained enrichment factor of HVA in the extract was 55. The possibility of HF-LPME of HVA was therefore proven.

4. Conclusions

Preliminary experiments proved possibility of HF-LPME of HVA and its subsequent voltammetric detection. Voltammetric sensing of HVA using miniaturized

three electrode system was successfully employed, with reference electrode inside Luggin capillary. For this purpose, a novel sensitive method of HVA determination at BDDE was developed, with limit of detection without the extraction step of 1.9 μmol dm^{-3} and limit of quantification 6.6 μmol dm^{-3}. Optimization of HF-LPME will follow.

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