Proceedings of the 13th International Students Conference "Modern Analytical Chemistry"

Prague, 21—22 September 2017

Edited by Karel Nesměrák

Charles University, Faculty of Science
Prague 2017

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Preface

The art of scientific communication is one of the most crucial parts of PhD. education. As many other abilities, it could be mastered only by practical writing, publicizing, and holding lectures. Our annual international students' conference "Modern Analytical Chemistry", is one of the ways how PhD. students of analytical chemistry could cultivate their presentation and communication skills. The conference was established in 2004, and from that time it is not only a forum for the presentation of achievements in the field of analytical chemistry by PhD. students from various countries but also nice social event.

This volume of the conference proceedings involves fifty one contributions, which were presented at the 13th year of conference on 21st–22nd September, 2017. 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 13th conference is one of the most successful years of the conference, as it was attended by 60 participants from six countries. We hope, that the attentive reader will find published contributions interesting and will be assured that – thanks to new generations of analytical chemists – analytical chemistry is trendy, multifaceted, 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.

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

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

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Contents

Contributions

Calibration problems in the acidity determination in wines

Paweł Świt*, Marcin Wieczorek, Joanna Kasperek, Paweł Kościelniak

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Keywords
Acidity
Analytical calibration
Flow analysis
Spectrophotometry
Wines

Abstract
The main objective of the research was to develop a calibration method allowing the spectrophotometric determination of total acidity of wines of different color (white, rose, and red) with improved accuracy and precision with the use of a designed flow system. The determination method was based on the reaction of analytes with a solution of bromothymol blue followed by measuring the absorbance at wavelength 615 nm. Nonlinear standard addition method with parabolic calibration function allowed compensation of the multiplicative interference effects. In addition, the additive effect caused by the wine color signal was also taken into account. As a consequence, the accurate and precise determination results of acidity have been obtained in all tested wines. The developed method provides shorter measurement time and lower consumption of reagents in comparison with recommended procedure – potentiometric titration.

1. Introduction

The conception of wine refers to an alcoholic beverage that is derived from the alcoholic fermentation process of fresh grape juice. Wines consist of many different components, primarily from water but also from alcohols (the highest amount of ethanol), organic acids, sugars, dyes, mineral salts and many other ingredients in smaller amounts [1].

Due to occurrence of a complex matrix of wine samples strong interference effects can appear. One type of interferences is the additive (unspecific) effect causing a constant change of analytical signal measured for the analyte. In addition, the effect known as the multiplicative one reflects linear (proportional) or nonlinear changes of the analytical signal with growth concentration of analyte. Tartaric acid in comparison with all considered acids is present in the highest amounts in wines and plays a very important role in providing chemical and microbiological stability. In accordance with the International Organization of Vine and Wine as well as the Association of Official Analytical Chemists the total

acidity of wines is a measure of the content of organic acids and is understood as the sum of titratable acids during neutralization the sample with the use of a base to the appropriate pH [2].

The total acidity of wines can be determined by titration of a wine sample with a strong base to the endpoint at pH = 7; the most common titrant is sodium hydroxide in presence bromothymol blue as an indicator. Alternatively, titration is also recommended to a pH = 8.2 in the presence of phenolphthalein [3] and potentiometric titration can also be distinguished [4].

One of the ways allowing compensation of the interference effects in investigated samples is the application of various calibration approaches. It can be distinguished commonly known way such as standard addition method (SAM). Nonlinear dependence between the analytical signal and the concentration of analyte in a context of SAM method can provide more accurate and precise analytical results. Calibration curve can be represented by different non-linear functions [5–7]. The application of SAM in nonlinear version is still not completely known and requires additional investigations.

2. Experimental

2.1 Reagents and samples

The following reagents were used: bromothymol blue (The British Drug Houses, UK); tartaric acid (Poch, Poland), disodium hydrogen phosphate dodecahydrate (Lach-Ner, Czech Republic), potassium dihydrogen phosphate (Poch, Poland), hydrogen peroxide 35% (Merck, Germany), ethanol 96% (Poch, Poland), sodium hydroxide 0.1 mol L^{-1} (Chempur, Poland). Distilled water derived from an HLP 5 system (Hydrolab, Poland) was used throughout.

The phosphate buffer of 0.2 mol L^{-1} was prepared by mixing 947 mL of 0.2 mol L^{-1} Na_2HPO_4 and 53 mL of 0.2 mol L^{-1} KH_2PO_4. The solution of 3.3×10^{-3} mol L^{-1} bromothymol blue was prepared by dissolving 0.206 g of bromothymol blue in 5 mL of ethanol and filling used as indicator. A 0.2 mol L^{-1} tartaric acid solution was prepared by dissolving of 3 g $C_4H_6O_6$ in 100 mL distilled water. The solution served for preparation of the synthetic sample of 0.020 mol L^{-1} and a set of standard solutions.

The following samples were investigated: Sophia Trakia white (Vinprom Byala, Bulgaria); Bordeaux rose (Producta, France); Fresco rose (Ambra S.A., Poland); Carlo Rossi rose (Carlo Rossi Vineyards, USA); Portada white (Jose Neiva Correia, Portugal); Carlo Rossi white (Carlo Rossi Vineyards, USA); El Sol red (Tierra de Castilla, Spain); Greenwood red (Vinemakers, USA).

2.2 Instrumentation

In order to determine the total acidity in wines, the flow-injection system shown in Fig. 1 was designed. It was consisted of two peristaltic pumps Minipuls 3

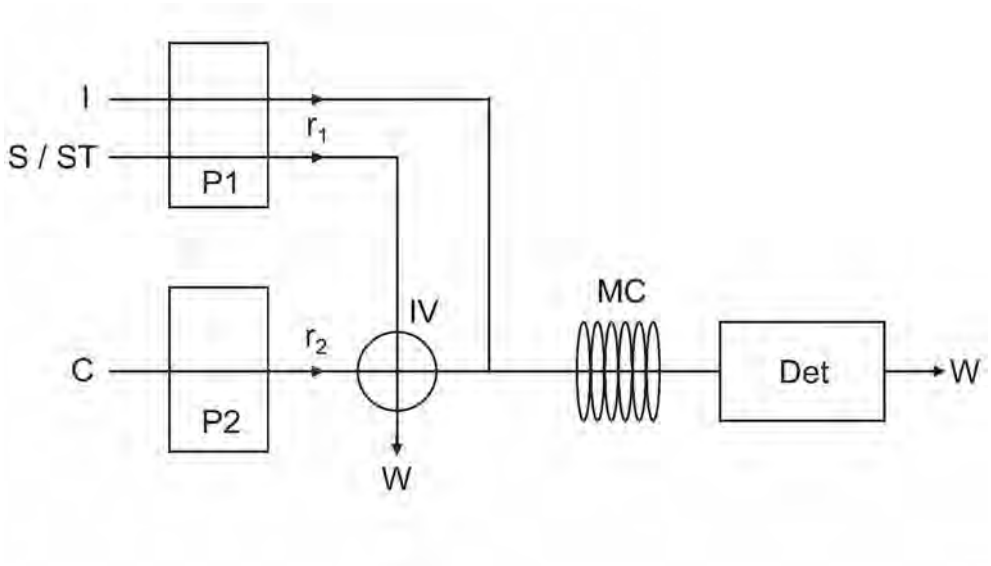

Fig. 1 Scheme of the flow-injection system: C – carrier, ST – standard, S – sample, I – indicator, P1 and P2 – peristaltic pumps, r_1, r_2 – flow rates, IV – injection valve, MC – mixing coil, W – waste, Det – detector.

(Gilson, France) and the injection valve (Gilson, France). Lambda 25 spectrometer (PerkinElmer, USA) equipped with 10 mm flow cell cuvette was used as the detector. Additionally, the following apparatus was used: 16 channel controller UVCTR-16 (KSP Elektronika Laboratoryjna, Poland) with software Valve and Pump Controller (KSP Electronics Laboratory, Poland), ultrasonic bath Sonic-3 (Polsonic, Poland), magnetic stirrer MS11 (Wigo, Poland).

The samples were titrated with the use of pH meter CP-501 (Elmetron, Poland) equipped with combined electrode ERH-11 (Hydromet, Poland).

2.3. Procedure

The sample were analysed with the use of the flow system presented in Fig. 1. This system worked in two modes: 1) injection loop was filled with a sample containing an acid component (analyte), whereas the carrier (distilled water), in combination with bromothymol blue was directed to the detector; 2) injection valve changed position and the sample solution was introduced into the carrier stream and directed to the detector. The signal measured at 615 nm was a discoloration of the bromothymol blue solution resulting in negative flow peaks on the basis of the reaction between the indicator and the analyte. An analytical signal was treated as the difference between the baseline signal and the minimum signal indicated by the flow peak. Each determination was repeated three times in the same experimental conditions.

Potentiometric titration with NaOH solution was used as a reference method. 20 mL of the wine samples were diluted to 60 mL and was titrated. Each assay was repeated three times and the result was determined using the Hahn method. The method was tested by titration of the synthetic sample (0.020 mol L^{-1} of tartaric acid) confirming the correctness of the method.

3. Results and discussion

Optimization studies were conducted on the basis of the synthetic sample (0.020 mol L^{-1} of tartaric acid). The following parameters were chosen: injection loop 100 µL, flow rate ratio r_1/r_2 = 1.0, mixing coil length 100 cm, flow rates 3.4 mL min^{-1}, and single peak time 40 s.

In the case of red and rose wines, the absorption of light at the selected wavelength was observed, which was the source of the additive interference effect. In the case of white wines, spectral interferences were not observed.

Conducted studies by an external calibration method have shown that in white and rose wines the multiplicative interference effect occurred (relative error between –20.0 and –30.0%). Ethanol, sulphur, and carbon monoxide were suspected to be potential interferents. The linear and non-linear standard addition method was used to compensate for these interferences.

In the presented studies the SAM method was used with various functions appropriately fitted to experimental results: linear and polynomial of degrees 2 and 3. Fig. 2 presents the calibration curves for synthetic sample dozed with four standard additions. The course of functions in the experimental area is similar, but diametrically different in the extrapolated area. As a consequence the analytical results, c_1–c_3, are drastically different from each other when various functions are used to describe the calibration dependence.

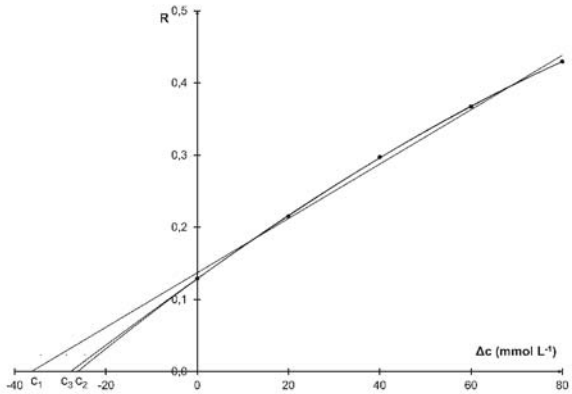

Fig. 2 Calibration curves obtained in the standard addition method for the synthetic sample; c_1, c_2, c_3 are analytical results calculated by extrapolation of linear, polynomial of degree 2, and polynomial of degree 3 functions, respectively.

Table 1
Estimation of the reference concentration, c_0, in various approximation ways: linear c_1, polynomial of degree 2, c_2, and polynomial of degree 3, c_3.

Sample	c_x / mmol L^{-1} (RE[%])			
	c_0	c_1	c_2	c_3
Synthetic without color	20.00	36.03 (80.1)	26.21 (31.1)	27.70 (38.5)
Sophia Trakia white	32.71	42.44 (29.8)	33.49 (2.4)	–
Bordeaux rose	48.49	55.97 (15.4)	45.02 (–7.2)	–
El Sol red	34.75	28.43 (–18.2)	19.02 (–45.3)	23.84 (–31.4)

In Table 1 results for synthetic sample and three real samples of wine: Sophia Trakia (white), Bordeaux (rose), and El Sol (red) are presented. The most inaccurate results with high relative error values compared to the reference concentration c_0 were obtained for linear approximation. Nonlinear approximation allowed the accuracy to be partly improved. However, it can be noticed that interference effects were still present in considered samples. Apparently, application of the SAM method in nonlinear version enabled compensation of only some part of interferences caused by a complex matrix of wines.

The colour of wine samples indicated occurrence of the additive effects. Then, for each sample the signal were measured prior to the chemical reaction (blank signal) and subtracted from the signal measured for this sample after reaction. Estimations of analytical results corrected in such a way using polynomial function of degree 2 are given in Table 2. As seen, total acidity of wines was determined with good accuracy (the relative error for most tested samples were not higher than 5%) and with very good precision (*RSD* = 2%).

Table 2
Final results of total acidity in investigated samples.

Sample	c_0 / mmol L^{-1} (RSD [%])	c_x / mmol L^{-1} (RSD [%])	RE [%]
Carlo Rossi white	44.96 (1.26)	44.51 (2.03)	–1.0
Sophia Trakia white	32.71 (0.74)	33.49 (1.64)	2.4
Portada white	37.76 (1.14)	36.06 (0.98)	–4.5
Fresco rose	50.52 (0.02)	49.91 (0.35)	–1.2
Bordeaux rose	48.49 (0.70)	52.00 (1.1)	7.2
Carlo Rossi rose	35.56 (0.39)	37.73 (1.82)	6.1
El Sol red	34.75 (0.76)	36.14 (1.19)	4.0
Greenwood red	38.25 (0.08)	39.05 (1.24)	2.1

4. Conclusions

The conducted study proved that the designed flow system enabled determination of total acidity in wines in a correct and repetitive way. The standard addition method with polynomial fitting allowed the multiplicative interference effects to be compensated, but the results obtained by this method were still different than the expected values. The source of remaining, additive effects was the color of the wine samples (particularly visible for red wines).

The flow method developed yielded satisfactory results in terms of accuracy and precision for all considered types of wine (white, rose and red). In addition, the analytical procedure is characterized by shorter measurement time and less reagent consumption compared to the reference method. The presented method can be recommended for routine determination of total acidity in wines.

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Voltammetric Determination of Tumor Biomarkers using Flow Injection Analysis with Amperometric Detection

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Keywords
flow injection analysis
5-hydroxyindole-3-acetic acid
homovanillic acid
vanillylmandelic acid
tumor biomarkers

Abstract
Three tumor biomarkers (homovanillic acid, vanillylmandelic acid, and 5-hydroxyindole-3-acetic acid) have been determined by flow injection analysis with amperometric detection at screen-printed carbon electrodes in optimum medium of Britton-Robinson buffer (0.04 mol l^{-1}, pH = 2.0). Dependences of the peaks current on the concentration of biomarkers were linear in the tested concentration region from 0.05 to 100 μmol l^{-1}, with the limits of detection of 0.065 μmol l^{-1} for homovanillic acid, 0.053 μmol l^{-1} for vanillylmandelic acid, and 0.033 μmol l^{-1} for 5-hydroxyindole-3-acetic acid (calculated from heights), and 0.024 μmol l^{-1} for homovanillic acid, 0.020 μmol l^{-1} for vanillylmandelic acid, and 0.012 μmol l^{-1} for 5-hydroxyindole-3-acetic acid (calculated from areas), respectively.

1. Introduction

Determination of homovanillic acid (4-hydroxy-3-methoxybenzeneacetic acid), vanillylmandelic acid (DL-4-hydroxy-3-methoxybenzeneacetic acid), and 5-hydroxyindole-3-acetic acid is a tool for prediction of neuroblastic and carcinoid tumors. Homovanillic and vanillylmandelic acids are major end products of catecholamine metabolism, 5-hydroxyindole-3-acetic acid is a breakdown product of serotonine (5-hydroxytryptamine) [1, 2]. These metabolites are excreted in urine, normal urinary concentrations being from 8.2 to 41.0 μmol l^{-1} for homovanillic acid, from 11.6 to 28.7 μmol l^{-1} for vanillylmandelic acid, and from 17.8 to 58.3 μmol l^{-1} for 5-hydroxyindole-3-acetic acid [3].

Increased urinary concentration can predict neuroblastoma and carcinoid syndrome. Apart of tumors, measuring of serotonine and 5-hydroxyindole-3-acetic acid in biological fluid can be useful for diagnosis hypertension, depression, migraine, and Tourette syndrome [4]. The most common methods for determination of 5-hydroxyindole-3-acetic acid in clinical laboratories are HPLC-ED and GC-MS [5]. Homovanillic acid, vanillylmandelic acid, and 5-hydroxyindole-3-acetic acid can be oxidized at carbon electrodes due to their phenolic structure [6]. In this work these compounds were determined at screen-printed carbon electrodes using flow injection analysis with amperometric detection.

2. Experimental

2.1 Reagents and chemicals

The stock solutions (1×10^{-3} mol l^{-1}) of tested compounds were prepared by dissolving appropriate amount of the substance in 100 ml of deionized water. All measurements were performed in Britton-Robinson buffer prepared in a usual way (i.e., by mixing a solution of 0.04 mol l^{-1} of phosphoric acid, 0.04 mol l^{-1} of acetic acid, and 0.04 mol l^{-1} of boric acid with the appropriate amount of 0.2 mol l^{-1} sodium hydroxide solution).

2.2 Instrumentation

Flow injection analysis was performed in apparatus depicted in Fig.1), consisting of syringe pump (NE-510L, LABICOM, CZ), injection valve (type 7725i, Rheodyne, California, USA) with 20 µl injection loop, flow cell (flow-cell in Teflon for screen-printed electrodes FLWCL-TEF, DropSens, Spain), and computer-con-trolled potentiostat PalmSens3 (PalmSens BV, Houten, The Netherlands). Screen--printed carbon electrodes used (type DRP-110, DropSens, Spain) were with a three-electrode system comprising of carbon working electrode (4 mm diameter), carbon counter electrode, and silver reference electrode.

For calculating calibration curve parameters and for graphic presentation of results, Microsoft Office Excel 2010 (Microsoft Corporation, USA) and OriginPro

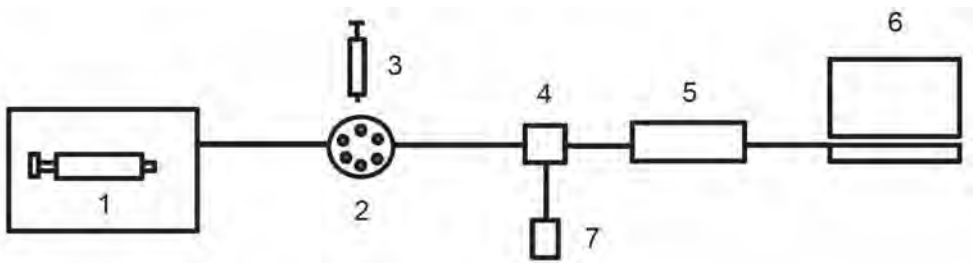

Fig. 1 Scheme of flow injection analysis apparatus: (1) syringe pump, (2) injection valve, (3) syringe for injection of samples, (4) flow cell, (5) potentiostat, (6) computer, (7) waste.

8.0 (OriginLab Corporation, USA) were used. The limits of quantification (*LOQ*) were calculated as $LOQ = 10s/a$, where s is the standard deviation of ten repetitive measurements of the lowest measurable concentration, and a is the slope of the calibration curve [7].

3. Results and discussion

To improve the background baseline, the influence of current range was tested. The best results were obtained when using the current range 1–100 μA. Therefore, all the measurements were performed at this current range.

In all cases, dependences of the peak height and the peak area on the tested parameter (potential of detection, flow rate, pH) were obtained and averages from three repetitive measurements were used in the graphs (not shown in this contribution). Flow rate was tested in the range of 0.5–2.5 ml min^{-1}, electrode potential in the range of 0.4–1.2 V, and pH in the range of 2.0–8.0. Parameters with highest response and/or lowest relative standard deviations were chosen as optimal. Under the optimum conditions, calibration curves for the peak height and the peak area in the concentration range 0.05–100 μmol l^{-1} were constructed. Flow injection analysis amperograms of 5-hydroxyindole-3-acetic acid are shown in Fig. 2 and Fig. 3 for the sake of illustration. Parameters of the calibration curves were evaluated and the obtained limits of detecion are summarized in Table 1.

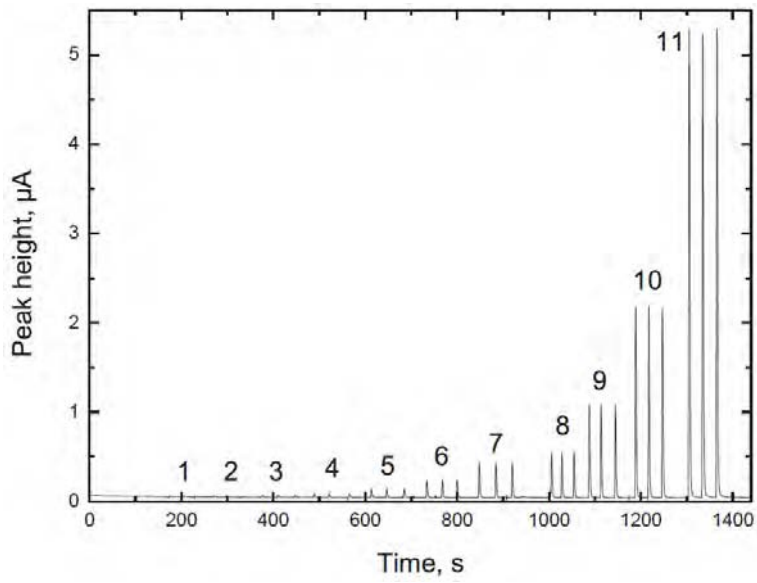

Fig. 2 Flow injection analysis with amperometric detection of 5-hydroxyindole-3-acetic acid on concentration levels: (1) 0.05 μmol l^{-1}, (2) 0.1 μmol l^{-1}, (3) 0.4 μmol l^{-1}, (4) 1 μmol l^{-1}, (5) 2 μmol l^{-1}, (6) 4 μmol l^{-1}, (7) 8 μmol l^{-1}, (8) 10 μmol l^{-1}, (9) 20 μmol l^{-1}, (10) 40 μmol l^{-1}, (11) 100 μmol l^{-1}, in Britton-Robinson buffer at pH = 2.0 on screen-printed carbon electrode, flow rate 1 ml min^{-1}, potential of detection +0.8 V, injected volume 20 μl.

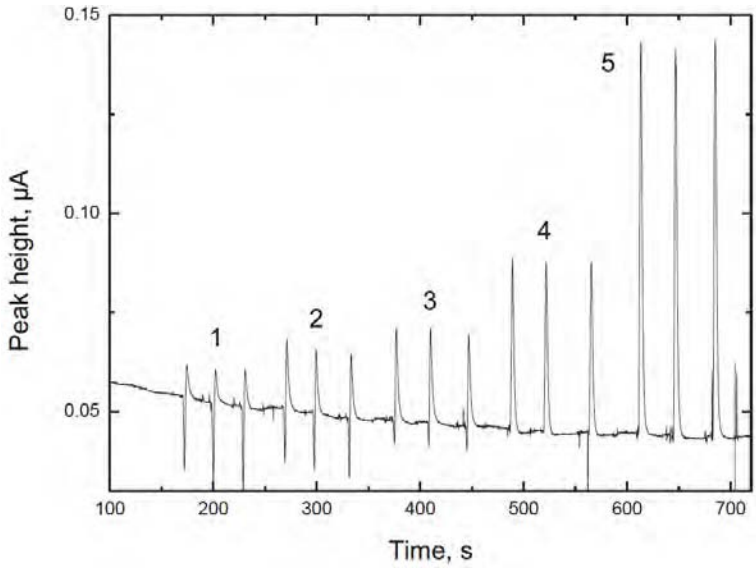

Fig. 3 Magnified flow injection analysis with amperometric detection recordings (from Fig. 2) of 5-hydroxyindole-3-acetic acid on concentration levels: (1) 0.05 µmol l^{-1}, (2) 0.1 µmol l^{-1}, (3) 0.4 µmol l^{-1}, (4) 1 µmol l^{-1}, (5) 2 µmol l^{-1}, in Britton-Robinson buffer at pH = 2.0 on screen-printed carbon electrode, flow rate 1 ml min^{-1}, potential of detection +0.8 V, injected volume 20 µl.

Table 1
Optimum conditions and figures of merits for flow injection analysis with amperometric detection determination of three tested tumor biomarkers. Current range 1–100 µA, flow rate 1 ml min^{-1}, pH = 2.0.

Analyte	Potential / V	LOD^a / µmol l^{-1}	LOD^b / µmol l^{-1}
homovanillic acid	+0.6	0.065	0.024
vanillylmandelic acid	+0.8	0.053	0.020
5-hydroxyindole-3-acetic acid	+0.8	0.033	0.012

[a] Calculated from peak heights.
[b] Calculated from peak areas.

4. Conclusions

Determination of homovanillic, vanillylmandelic, and 5-hydroxyindole-3-acetic acids is important for prediction and diagnosis some diseases, including neuroblastic and carcinoid tumors and could help to start their treatment in time.

This work describes FIA with amperometric detection as a suitable method for the determination of these tumor biomarkers. Britton-Robinson buffer was used as mobile phase. Optimum parameters were found and under these conditions calibration dependences were measured. Calibration dependences were linear in the whole tested concentration range from 0.05 to 100 µmol l^{-1} and their

parameters were evaluated and *LOD*s were found. There is no problem with passivation using flow injection analysis with amperometric detection and further research could be focused on the determination of tumor biomarkers in human urine, probably with preliminary separation, preconcentration, and solid phase extraction.

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A New Method for Detection and Classification of Non-Volatile Nitroso Compounds in Beer Combining Gas Chromatography with Chemiluminescence Detection and Discriminant Analysis

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Keywords
beer
chemiluminescence detection
discriminant analysis
gas chromatography
nitroso compounds

Abstract
The problems of beer contamination with nitroso compounds have been addressed since 70´s of twentieth century and have been partially solved, namely concerning the contamination by carcinogenic volatile *N*-nitrosamines. However, there is still a gap in the knowledge of non-volatile nitroso compounds in terms of both the determination of these compounds and description of their toxicity. Therefore, a new method for their detailed study is necessary. Based on these facts, a new method permitting the detection and classification of non-volatile nitroso compounds groups in beer, such as *N*-nitroso, *C*-nitroso, and interfering substances. The method is based on profiling of pyrolytic products of non-volatile nitroso compounds represented by GC chromatographic peaks determined by chemiluminescence detector and subsequent multivariate chemometric classification. The resulting classification function shows a good classification performance with total accuracy of 96.12% after method validation. The method was applied and demonstrated on microbiologically contaminated beer sample.

1. Introduction

Nitroso compounds, specifically carcinogenic *N*-nitrosamines, are well-known contaminating compounds in beer. These compounds can be divided into volatile and non-volatile ones and it is possible to determine these two groups as their apparent total concentration: Apparent Total *N*-nitroso Compounds (ATNC) in μg(N-NO)/kg [1]. Nitroso compounds can be further classified into *C*-, *N*-, *S*-, and *O*-nitroso compounds. Sources of nitroso compounds in beer are malt and

bacterial contamination of brewing intermediates and/or final product. Whereas the problem of the nitroso compounds originating during the malting process was nearly eliminated due to technological changes, the risk of a high amount of ATNC in beer via microbial contamination still persists. Volatile *N*-nitrosamine concentrations in beer are very low, even after microbial contamination, e.g. *N*-nitrosodimethylamine concentration is usually < 0.2 μg/kg (expressed as ATNC < 0.12 μg(N-NO)/kg) [1]. Based on our previous study [2], the common concentration of ATNC in beer is < 20 μg(N-NO)/kg and only about 1 % of ATNC in beer is formed by volatile *N*-nitrosamines, non-volatile ones representing the vast majority of total ATNC in beer. Despite this fact, nobody has as yet described the spectrum of ATNC compounds in more detail.

Volatile *N*-nitrosamines are routinely determined by gas chromatography with nitrosamine specific chemiluminescence detection (GC-NCD) [3]. However, a routine and reliable analytical method for determination of non-volatile *N*-nitrosamines doesn't currently exist because the structure of these compounds has not yet been elucidated. The presence of non-volatile nitroso compounds in beer is evident from the comparison of volatile *N*-nitrosamine concentrations and ATNC concentration determined by denitrosation reaction by hydrogen bromide in acetic acid solution, with consequent quantification of released nitric oxide by NCD [4].

From the nature of compounds present in beer (relatively high concentration of polyphenolic compounds) it is likely that part of ATNC is formed by *C*-nitroso compounds arising by an interaction of phenolic compounds with nitrosation agents produced by bacterial contamination, and their formation inhibits *N*-nitrosamine formation [5, 6].

Any conclusion about the health risk arising from the intake of non-volatile nitroso compounds in beer cannot be made without detailed knowledge of their chemical structure. A possible way to search for non-volatile nitroso compounds is to detect them by NCD after derivatization of polar groups and GC separation. However, the problem is that NCD is not selective enough and some false positive peaks are common in the chromatogram. These peaks could be falsely identified as nitroso compounds. Therefore, the classical NCD is blind in searching for unknown non-volatile constituents of ATNC.

For these reasons, there is requirement for a method which will be able to find unknown non-volatile compounds as individual chromatographic peaks for subsequent detailed structural elucidation.

The aim of this work is to develop a method for classification of non-volatile nitroso compounds in beer into different nitroso compound types and distinguish them from interfering com-pounds by GC with pyrolytic profiling NCD. This method uses changes in peak intensity depending on NCD pyrolytic temperature and multivariate chemometric evaluation. The workflow of the method is schematically described in Fig. 1 (next page).

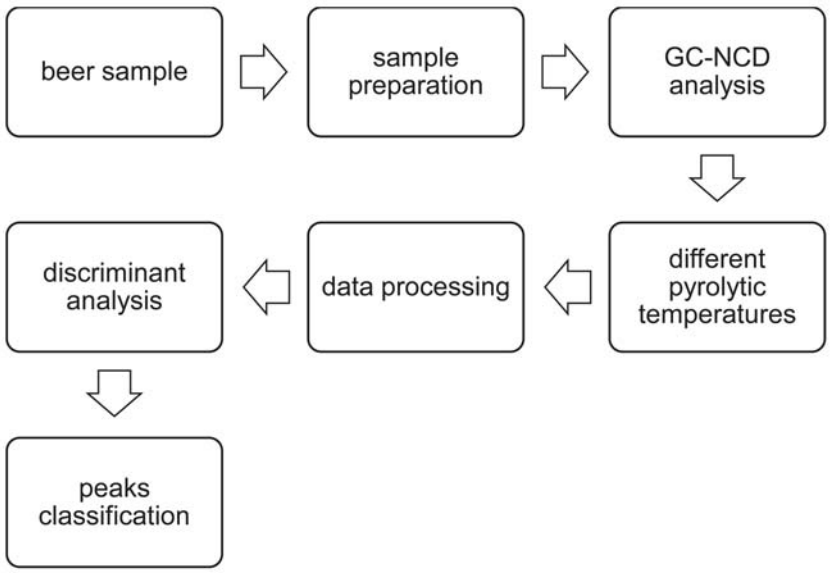

Fig. 1 Applied workflow for classification of nitroso compounds in beer.

2. Experimental

2.1 Reagents and chemicals

The following substances were used for construction of classification function by discriminant analysis: methanolic solutions of *N*-nitrosamines, namely *N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitrosodiisopropylamine, *N*-nitrosodipropylamine, *N*-nitrosodibutylamine, *N*-nitrosopiperidine, *N*-nitrosopyrolidine, *N*-nitrosomorpholine, *N*-nitrosomethylphenylamine, *N*-nitrosoethylphenylamine (100 μg/ml, Ultra Scientific, USA), *N*-nitrosodiethanolamine solution (100 μg/ml, Neochema, Germany), *N*-nitrosoproline, *N*-nitrososarcosine, *N*-nitrosopipecolic acid, and *N*-nitrosonornicotine (100 μg/ml, Isconlab, Germany); *C*-nitroso, nitro and other interfering compounds, namely nitrosobenzene (95%, Merck, Darmstadt, Germany), nitrosotoluene (97%), *N,N*-dimethyl-4-nitrosoaniline (97%), 2-nitroso-1-naphtol (95%), methoxy-3-nitro-2--nitrosobenzene (98%), nitroethane (96%), nitropropane (98%), 2,4-dinitrophenol (95%), nitrobenzene (> 99%), 2-nitroaniline (99%), eugenol (99%), 2-ethylphenol (99%), 2-allylphenol (99%), aniline (> 99.5%), benzonitrile (99%), 2-furankarbaldehyde (99%), 3,4-dihydroxybenzoic acid (97%), pyridine (≥ 99.8 %), catechine (99%, Sigma-Aldrich, Germany), gramine (99%, Serva, Germany). Solution of *NO*-bis-trimethylsilyl-trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (99:1, *v/v*, Supelco, Germany) were used for derivatization of non-volatile compounds. Solution of ammonium amidosulphonate (99 %, Merck, Germany) and sulphuric acid (96%, Lachner, Czech Republic)

was used as nitrite scavenger (see section 2.4). The following solvents were also used: acetonitrile (≥ 99.9%) and methanol (≥ 99.9 %, both from Sigma-Aldrich, Germany). Deionized water was produced by Milli-Q system.

N-nitramines were prepared by oxidation of corresponding *N*-nitrosamines in dichloromethane solution (2 ml, 50 µg/ml) by 1 ml trifluoroacetic anhydride (Merck, Germany) and 0.6 ml hydrogen peroxide (33%, Merck, Germany) at 65 °C for 60 minutes and were recrystallized in 5 ml ethanol (96%, Lachner, Czech Republic) [7].

2.2 Instrumentation

Gas chromatographic analyses were carried out by Thermo Trace 1310 gas chromatograph equipped with TG-200MS capillary column (30 m, 0.25 mm ID, 0.25 µm film thickness of trifluoropropylmethyl polysiloxane stationary phase). Injection of 1 µl sample solution was performed by split injection technique at split ratio 1:10 at 210 °C. Flow of argon (Messer, 99.996%) as a carrier gas was maintained at 0.6 ml/min. Programmed oven temperature during analysis was set as follows: 50 °C (1.5 min) – 20 °C/min – 150 °C (5 min) – 10 °C/min – 210 °C (3 min) – 10 °C/min – 320 °C (10 min). Chromatographic zones were detected by Ellutia 820 TEA chemiluminescence detector at different temperatures of pyrolytic tube to obtain pyrolytic profiles (500, 600, 650, 700, 750, and 800 °C). The flow of oxygen as a reagent gas for ozone generation in the detector was set at 3.2 ml/min.

2.3 Derivatization

Non-volatile standard compound solutions were dried under stream of nitrogen and 150 µl of derivatization reagent (BSTFA + TMCS) was added. After 15 minutes at laboratory temperature, samples were directly analysed by GC-NCD. Dried real samples (after sample preparation, see section 2.4) were derivatized in the same way as in the case of standard solutions.

2.4 Beer sample preparation

Beer samples were pre-treated by precipitation and SPE clean-up. Samples were first degassed in ultrasonic bath for 20 seconds. Six millilitres of sample was mixed with 1 ml of 0.2 mol/l ammonium sulphonate in 0.2 mol/l sulphuric acid to scavenge nitrites in the sample. The sample was then precipitated by 24 ml of acetonitrile and allowed to sediment overnight in a fridge. Supernatant was then transferred into a heart shaped flask and acetonitrile was distilled from the sample on vacuum evaporator (45 °C, 100 mbar). Remaining solution was passed through a solid phase extraction tube (Strata C-18E, 500 mg, Phenomenex) conditioned by 6 ml of methanol and 6 ml of deionized water. The solution passed

Table 1
Pyrolytic temperatures and their ratios selected for classification function.

t / °C									
500	650	800	800/700	800/650	700/500	750/500	800/500	700/500	750/500

through the column was evaporated to dryness on vacuum evaporator (45 °C, 30 mbar). The residues were redissolved in 100 µl acetonitrile and 100 µl methanol, both were transferred into vials with an insert and evaporated to dryness under the stream of nitrogen. Samples were derivatized as mentioned in section 2.3.

2.5 Data processing

Different classes of compounds with positive response to NCD in the range of pyrolytic temperature from 500 to 800 °C give different pyrolytic profiles. In order to construct the classification function the relative peak areas (related to the highest area across the pyrolytic profile) and pairwise ratio between these areas of each standard compound (at least two different concentrations) were used as independent variables for linear discriminant analysis. Dependent variables were classes of compounds as follows: *N*-nitroso, *C*-nitroso, combination of *C*-nitroso and nitro, *N*-nitro and interferences including nitro compounds and non-nitroso aromatic compounds. Forward stepwise selection method was used to choose the most discriminating variables for final discriminant function (tolerance 0.01). The most discriminating variables selected for reliable classification are given in Table 1. These variables were further used as independent variables in discriminant classification. The data set (n = 102) was randomly divided into training and test set (1:1). The training set was used to construct discriminant function and its performance was checked by the test set as well as leave-one-out validation method.

3. Results and discussion

3.1 Performance of discriminant analysis classification

Resulting discriminant analysis displayed in first three canonical variables shows sufficient separation of compound classes (Fig. 2). Fitting the data with classification function gives 100 % accuracy (percentage of correctly classified objects). Classification performance was evaluated by the test set and leave-one-out validation method. Results from classification of test set samples show accuracy of 98.04% (only one sample was incorrectly classified). Results from leave-one-out method are summarized in confusion matrix (Table 2) with total accuracy of 96.12%. The worst accuracy was obtained for *C*-nitroso group due to misclassification of three objects into *N*-nitro group, because these two groups are very close

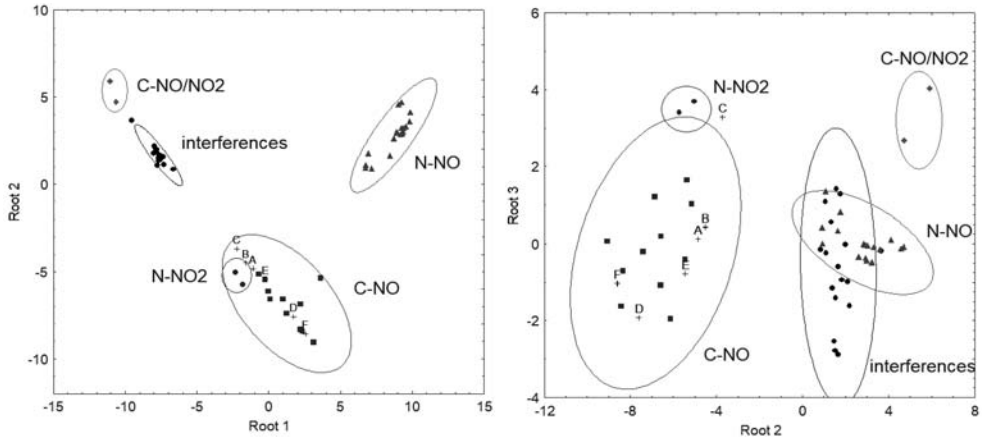

Fig. 2 Discriminant analysis canonical scores of pyrolytic profiles from different classes of compounds. Zoomed peaks from Fig. 3 are marked as black crosses.

Table 2
Confusion matrix obtained by leave-one-out cross-validation method.

Class	Correct / %	*C*-nitroso	*N*-nitroso	interferences	*C*-nitroso/nitro combination	*N*-nitro
C-nitroso	76.92	13	0	0	0	3
N-nitroso	100.00	0	35	0	0	0
Interferences	97.62	0	0	42	1	0
C-nitroso/nitro combination	100.00	0	0	0	6	0
N-nitro	100.00	0	0	0	0	3
Total	96.12					

in multidimensional space; this can be seen in discriminant analysis canonical scores in Fig. 2. To evaluate model performance from a statistical point of view the model accuracy should be compared with accuracy given by random assignation of objects to one of the defined classes [8]. In this case the random accuracy is 32.47% and it is lower than model accuracy; the obtained classification model can therefore be considered as a model with good performance.

3.2 Real sample application

The method was applied and its use demonstrated on beer sample artificially contaminated by *Escherichia coli*, which is a known nitrate reducing bacterium and producer of nitroso compounds [9]. The sample was treated according to the method. To verify the classification results and compare nitroso compounds originating from malt and from bacterial contamination, the chromatogram

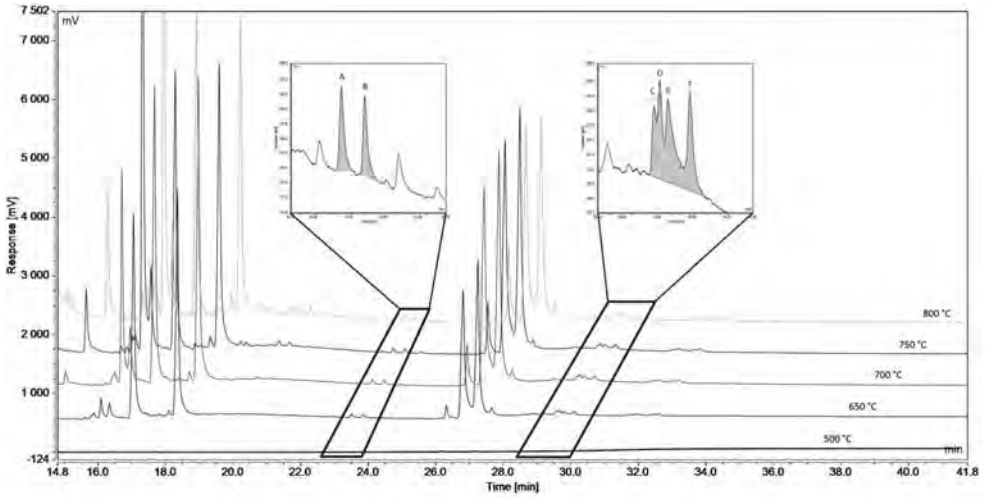

Fig. 3 GC-NCD chromatogram of artificially bacterially contaminated (*Escherichia coli*) beer sample at different pyrolytic temperatures. Nitroso compounds produced by contamination are zoomed at pyrolytic temperature 750° C.

of bacterially contaminated sample was compared with original non-contaminated sample. GC-NCD chromatogram of contaminated beer sample with zoomed peaks of classified nitroso compounds from bacterial contamination is given in Fig. 3. These peaks are also displayed as black crosses in Fig. 2; all of these unknown peaks were classified as *C*-nitroso compounds.

4. Conclusions

A method for classification of NCD chromatographic peaks for non-targeted nitroso compound analysis with good performance was developed. This method is suitable for detection and classification of nitroso compounds into the *C*- and *N*-nitroso group and their distinguishing from other interfering compounds. It is the first and significant step in identifying unknown non-volatile nitroso compounds in beer. The method was demonstrated on an artificially microbiologically contaminated beer sample. The great majority of nitroso compounds detected were classified into *C*-nitroso group. The method is also usable for malt samples or other food matrices.

Acknowledgments

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Isotachophoretic Determination of Amino Acids After Their Conversion to Hydroxy Acids

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Keywords
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hydroxy acids
isotachophoresis

Abstract
Determination of amino acids by capillary isotachophoresis is presented. Developed method is suitable for separation of amino acids from relatively simple mixtures, especially for dietary supplements. The most of amino acids is not possible to separate directly by isotachophoresis. Thus, they had to be converted to other compounds. Chemical conversion to corresponding hydroxy acids in presence of nitrite acid was used. This necessary nitrite acid was prepared by reaction of sodium nitrite with acetic acid. Under optimization, concentration 0.1 mol/L sodium nitrite and acetic acid was found. As the best electrolyte system for separation of hydroxy acids, 0.01 mol/L HCl, 0.05% hydroxyethyl cellulose, with β-alanine (leading electrolyte of pH = 3.6) and 0.01 mol/L valeric acid with sodium hydroxide (terminating electrolyte of pH = 7.24) were chosen.

1. Introduction

Nowadays, it is generally known that consumers, producers, and supervisory bodies have raised interests to know which ingredients and nutrients are present in food. According to this, a lot of methods for determination of various nutrients are developed. This study was focused on determination of amino acids. These biologically active compounds are necessary for human nutrition because they represent the basic building component of proteins.

Many various methods for determination of amino acids exist. High-performance liquid chromatography and capillary electrophoresis are the most popular separation techniques for simultaneous determination of amino acids. Moreover, several papers also deal with isotachophoretic determination of amino acids [1–3].

Generally, amino acids are ionized in water solutions, but their molecules are almost electroneutral due to presence of positive and negative charge. For that reason, mobility of amino acids is low in electric field. This phenomenon causes

impossibility of direct separation. There are some exceptions: amino acids with two –COOH groups (glutamic and aspartic acid) and basic amino acids (histidine, lysine, and arginine) can be separated by isotachophoresis without pre-treatment, because their mobility in electric field is high enough.

One of the possibilities how to separate amino acids by isotachophoresis can be their conversion to hydroxy acids using nitrite acid

$$HOOC–CHR–NH_2 + HNO_2 \rightarrow HOOC–CHR–OH + N_2 + H_2O \quad (1)$$

This reaction of primary amino groups [4] was used.

2. Experimental

2.1 Reagents and chemicals

All used amino acids (L-valine, L-leucine, L-isoleucine, L-tyrosine, L-lysine, L-tryptophan, L-arginine, L-histidine, L-cysteine, L-serine, L-alanine, glycine, L-phenylalanine, L-asparagine, L-glutamine, L-glutamic acid, L-aspartic acid, L-threonine, L-proline) and α-cyclodextrine were purchased from Sigma-Aldrich (USA). Sodium nitrite, acetic acid (99%), sulfuric acid (98%), hydrochloric acid (36%), valeric acid, and sodium hydroxide was purchased from Lachema (Czech Republic). Hydroxyethyl cellulose (4000) and buffer β-alanine were purchased from Serva (Germany). Deionized water was used to dilution of solutions.

2.2 Amino acids conversion

Amino acid (less than 1 mmol/L) was quantitatively transfered into 50 mL volumetric flask. Additionally, 5 mL of 1 mol/L sodium nitrite and 5 mL of 1 mol/L acetic acid were added into the same volumetric flask. In the end, whole flask was filled in by deionized water. It was placed on the plate of magnetic stirrer and boiled in water bath at 100 °C for 10 minutes. After that, this solution was twenty times diluted before isotachophoretic analysis.

2.3 Instrumentation

EA 102 (Villa-Labeco, Slovakia) was used as isotachophoretic analyser. This analyser is equipped with two polytetrafluorethylene capillary columns: pre-separation (160×0.8 mm) and analytical (160×0.3 mm). Individual zones are detected by conductivity detector. Isotachophoreograms were evaluated in a software ITP Pro supplied with the analyser.

Analysis was performed in anionic mode with leading electrolyte consisting 10 mmol/L hydrochloric acid, 0.05% hydroxyethyl cellulose, with β-alanine (pH = 3.6) and terminating electrolyte consisting 10 mmol/L valeric acid with

sodium hydroxide (pH = 7.24). Separation of leucine and isoleucine was performed in anionic mode with electrolyte system consisting 10 mmol/L hydrochloric acid, 20 mmol/L α-cyclodextrine, with β-alanine as leading electrolyte of pH = 3.4 and 20 mmol/L acetic acid as terminating electrolyte. The driving current used for the pre-separation capillary was 250 μA and for the analytical capillary 50 μA, respectively. During detection, last mentioned current was decreased to 30 μA. Each analysis required maximally 45 min.

3. Results and discussion

3.1 Conversion of amino acids to hydroxy acids

Working conditions necessary for amino acids conversion had to be optimized. Whole optimization was done on non-polar amino acid leucine.

Concentration of sodium nitrite was the first parameter which was optimized. Six concentrations of sodium nitrite were selected, such as 10, 20, 50, 100, 200, and 400 mmol/L. For achievement of acidic conditions to form nitrite acid, 100 mmol/L acetic or 100 mmol/L sulfuric acid was used. From Fig. 1 it is evident that acetic acid represents better choice because conversion efficiency was higher by 15% at acetic acid. The lowest concentration of sodium nitrite to achieve the highest efficiency was 100 mmol/L for acetic acid. However, maximal efficiency about 85% at concentration 200 mmol/L sodium nitrite was found for sulfuric acid.

Selection of suitable acid was the next optimized parameter. Sulfuric, acetic, and valeric acid were tested. Dependence of leucine conversion efficiency on these acids of concentrations 5, 10, 20, 40, 100, 200, and 400 mmol/L was measured. It was found that sulfuric acid is not suitable for this purpose, because maximum

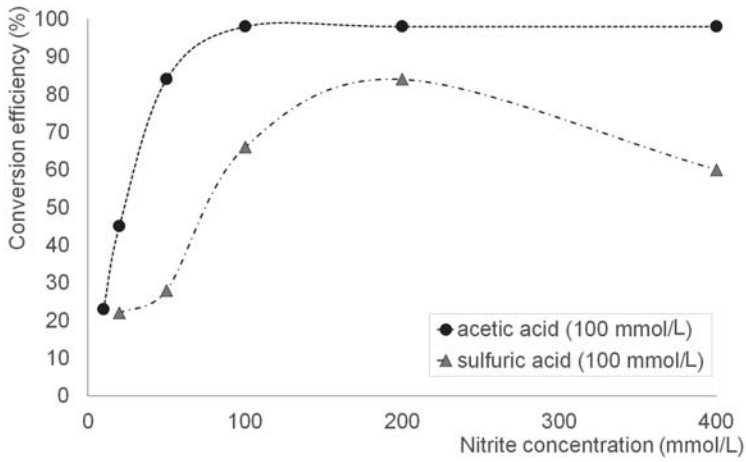

Fig. 1 Dependence of leucine conversion efficiency on sodium nitrite concentration for acetic and sulfuric acid.

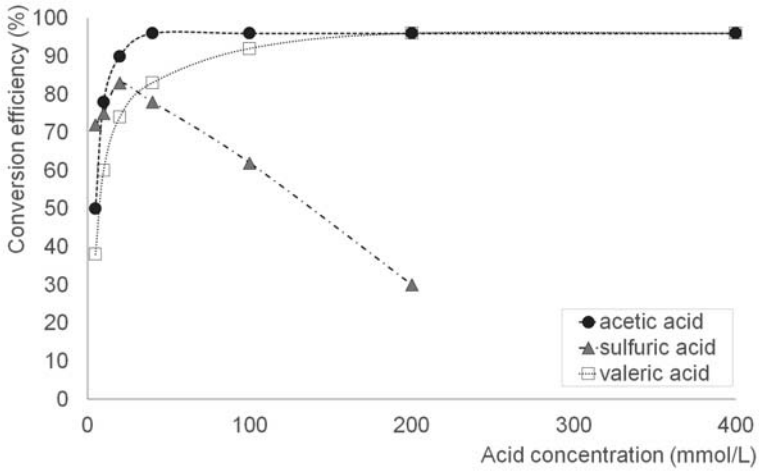

Fig. 2 Dependence of leucine conversion efficiency on acid concentration for different acids (concentration of sodium nitrite 100 mmol/L).

of conversion efficiency was about 85 %. Moreover, higher concentration than 20 mmol/L sulfuric acid significantly decreased the efficiency, what is clear from Fig. 2. The conversion efficiency using acetic, and valeric acid was much better (about 99% for both of them) than in previous case. Optimum concentration of acetic acid was 50 or 100 mmol/L and of valeric acid 200 mmol/L to achieve maximum efficiency about 99%. One of other significant advantages is fact that these acids are also composition of terminating electrolyte.

At laboratory conditions, the kinetic of conversion is very slow. Evidently, yields of this chemical reaction would be very low, which could significantly affect the sensitivity of the final analytical method. Ways how to increase the reaction rate in the favor of corresponding products were tested.

Mixture of leucine solution, 100 mmol/L sodium nitrite, and 100 mmol/L acetic acid was only stirred for different times. After certain time (2, 5, 10, 20, 50, 100, 200 minutes), 5 mL of reaction mixture were twenty times diluted, analysed by isotachophoresis and conversion efficiency was measured.

Moreover, the same experiment was repeated with one exception where the stirring was replaced to ultrasonic bath. As the last combination, stirring of boiled reaction mixture was used. The best conversion efficiency about 99% was achieved by stirring and boiling together for time duration longer than 10 minutes, what is demonstrated in Fig. 3 (next page). Unfortunately, itself stirring or using of ultrasonic bath was not sufficient; efficiency lower than 90%.

3.2 Isotachophoretic separation

Isotachophoretic separation of twenty encoded amino acids after conversion to corresponding hydroxy acids was performed in already mentioned anionic mode.

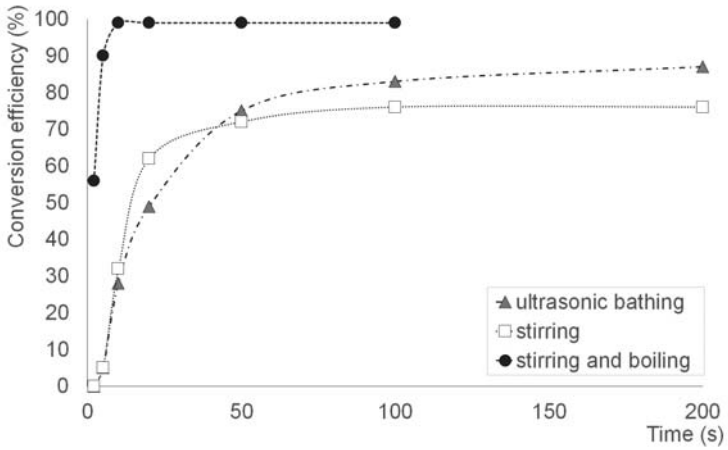

Fig. 3 Relationship between leucine conversion efficiency and time for different reaction supports.

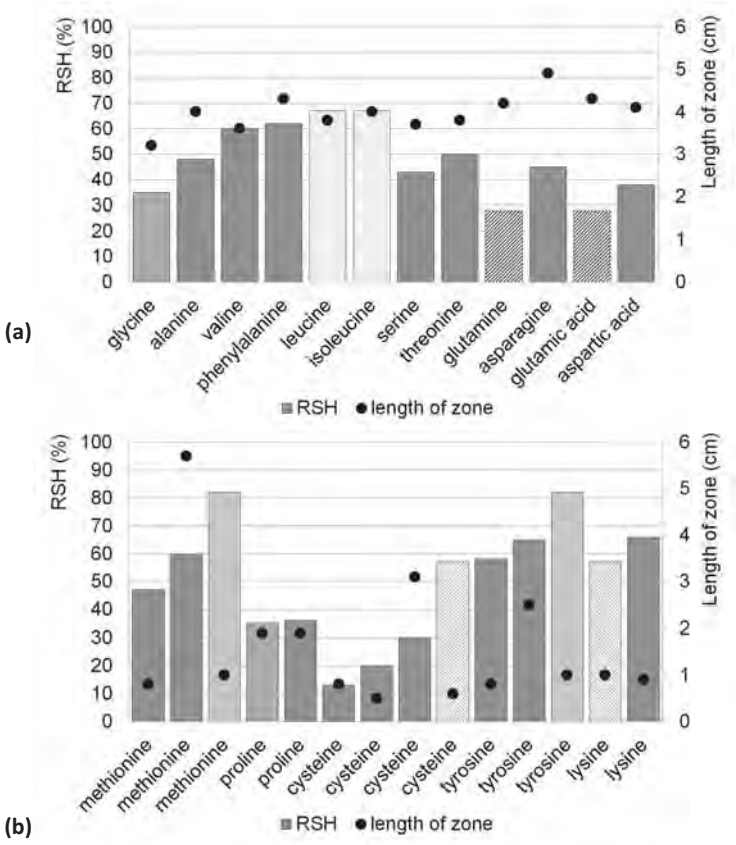

Fig. 4 Relative signal highs (RSHs) and lengths of zones in analytical capillary of (a) amino acids (0.25 mmol/L) with one conversion product, and (b) amino acids (0.25 mmol/L) with the more conversion products (two, three or four).

The most of amino acids provided minimally one isotachophoretically determined product. However, it should be state that reality is more complicated as follow: proline and lysine (two), methionine and tyrosine (three), cysteine (four), and tryptophan, arginine and histidine (no products).

In determination of amino acids providing only one measured product (Fig. 4a), different heights of signal were obtained for each product. Leucine with isoleucine and glutamic acid with glutamine were the exceptions. Glutamic acid and glutamine gave the same product: 2-hydroxyglutaric acid. Leucine and isoleucine after conversion is possible to separate with some addition of α-cyclodextrine to leading electrolyte. Therefore, several electrolyte systems with different concentration of α-cyclodextrine in the leading electrolyte were tested. As the best choice, 10 mmol/L hydrochloric acid, 20 mmol/L α-cyclodextrine, and β-alanine (pH = 3.4) was selected. 20 mmol/L acetic acid was used as terminating electrolyte.

Effect of presence of α-cyclodextrine in the leading electrolyte is evident in the Fig. 5. With the higher concentration of α-cyclodextrine increased the detector response of leucine, isoleucine, and also of terminating electrolyte. Optimum amount of α-cyclodextrine for separation of isoleucine and leucine were chosen based on the maximum distances.

However, it should be mentioned that amino acids having more than one conversation product (Fig. 4b) complicate the analysis because some products create mixed zone (products which create mixed zone have the same pattern in Fig. 4). For that reason, developed method is not suitable for analysis of all encoded amino acids. Fortunately, relatively simple samples, especially dietary supplements, can be analyzed by isotachophoresis.

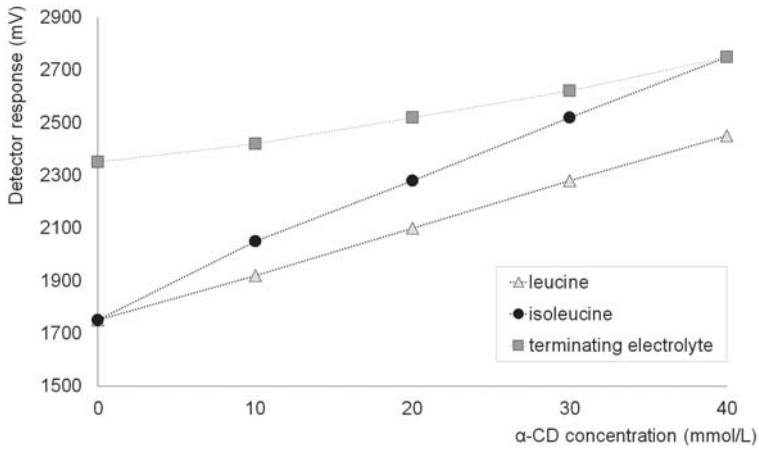

Fig. 5 Dependence of conductivity detector response on α-cyclodextrine concentration in leading electrolyte (pH = 3.4).

4. Conclusions

Isotachophoretic determination of amino acids converted to corresponding hydroxy acids was done. Optimum concentration of sodium nitrite and acetic acid was 0.1 mmol/L. As the more suitable way necessary for amino acids conversion, boiling in water bath for 10 minutes with simultaneous stirring was used. Under these conditions, the conversion efficiency was almost 100 %. Satisfactory separation of hydroxy acids was observed in the 0.01 M HCl, 0.05% hydroxyethyl cellulose and β-alanine (pH = 3.6) as the leading electrolyte and 0.01 M valeric acid and sodium hydroxide (pH = 7.24) as the terminating electrolyte. To conclude, it can only be said that developed method is not difficult for sample preparation and is completely suitable for determination of amino acids in the various food supplements.

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HPLC Separation Using Cyclofructan Selectors With Emphasis on Enantiomer Elution Order

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Keywords
cyclofructan
elution order
enantioseparation
HPLC

Abstract
The separation of enantiomers biologically active analytes was studied on cyclofructan chiral high-performance liquid chromatography columns in normal-phase mode. The effects of column temperature on retention, enantioseparation, and elution order were investigated. The reversal of enantiomer elution order was observed depending on the chemistry of the chiral selector.

1. Introduction

Chiral high-performance liquid chromatography (HPLC) is an effective tool for deter-mining optical purity. In this case, enantiomer elution order is an important issue in chiral separations from both, practical and theoretical points of view. When the enantiomeric impurity has to be analyzed in the presence of the major enantiomer, it is always desirable to elute the minor component in front of the major one [1]. For example, even an enantiomeric impurity as high as 1% was often impossible to detect when it is eluted as the second peak.

Several types of elution order reversal between enantiomers in chiral HPLC have been found [2]. The first type is temperature induced reversal of elution order. In 1984 Koppenhöfer and Bayer predicted the existence of an isoenantioselective temperature based on the van't Hoff equation. Below isoenantioselective temperature, separation is enthalpy driven, and the selectivity decreases with increasing temperature. Above isoenantioselective temperature, enantioseparation is entropy driven, and a reversal of the elution order for a pair of enantiomers is expected [3]. Temperature induced the reversal of enantiomer elution order on polysaccharide-based chiral stationary phases have been reported in the literature [1, 4, 5]. The second type of reversal of elution order is solvent induced. Not only the type [6] but also the content of modifier [7] and the percentage of minor acidic and basic additives in the mobile phase [8] can induce the reversal elution order. The third type is the reversal of enantiomer elution order depending on the type of the chiral selector [1, 9].

Cyclofructans (CFs) are a relatively new group of chiral selectors belonging to the macrocyclic oligosaccharides. They consist of six or more β-(2→1) linked D-fructofuranose units. Their abbreviations CF6, CF7, etc. indicate the number of fructofuranose units in the macrocyclic ring. Each fructofuranose unit contains four stereogenic centers and three hydroxyl groups, which can be utilized for derivatization.

The goal of the present work was the study of enantioseparation of spirobrassinin derivatives on cyclofructan chiral columns under normal phase conditions with emphasis on differences in enantiomer elution order and its dependence on the nature of the chiral selector and column temperature.

2. Experimental

2.1 Reagents and chemicals

The series of spirobrassinin derivatives, either in racemic form or in enantiopure form, were synthesized at the Department of Organic Chemistry, P. J. Šafárik University, Košice, according to the procedure described in the literature [10].

HPLC grade organic solvents *n*-hexane, and 2-propanol were purchased from Sigma-Aldrich (USA), while ethanol and analytical grade purity trifluoroacetic acid (99.8% purity) were purchased from Merck (Germany). Stock solutions of analytes (50 μg mL^{-1}) were prepared in 2-propanol or ethanol in dependence on mobile phase used.

2.2 Instrumentation

Chromatographic analyses were performed on the 1260 Infinity HPLC system (Agilent Technologies, Germany) consisting of a solvent degasser, a quaternary pump, an injection valve Rheodyne model 7125 (Cotati, CA, USA) with a 20 μL loop, a column oven, and an UV/ VIS detector with variable wavelength. The collection and evaluation of data were carried out using Agilent ChemStation software.

The chromatographic columns Larihc CF6-RN, Larihc CF7-DMP, and Larihc CF6-P are based on derivatized cyclofructans, namely R-naphthylethyl carbamate cyclofructan 6 (CF6-RN), dimethylphenyl carbamate cyclofructan 7 (CF7-DMP), and isopropyl carbamate cyclofructan 6 (CF6-P) immobilized on 5 μm silica gel support, all 250 mm × 4.6 mm i.d. were obtained from the Department of Chemistry and Biochemistry, University of Texas at Arlington (USA). The columns were operated at 25 °C except for the temperature effect study. The flow rate was 0.8 mL min^{-1} for all measurements. UV detection was performed at 250 nm.

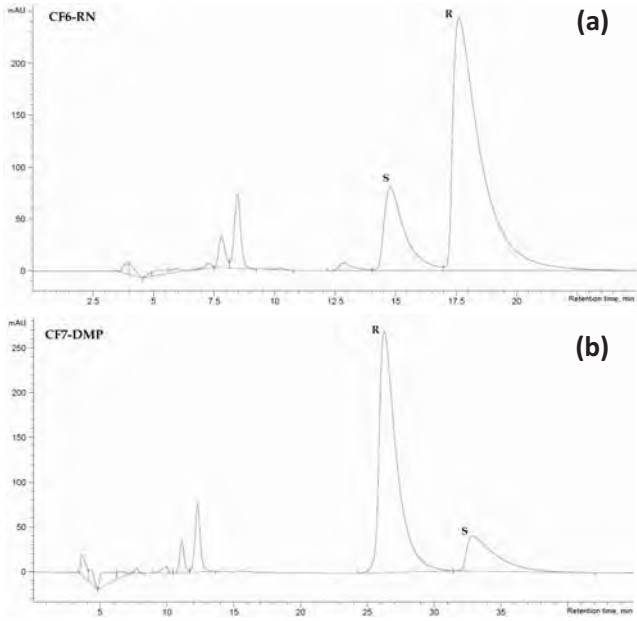

Fig. 1 Enantioseparation of selected synthesized enantiomer on (a) CF6-RN, and (b) and CF7-DMP. Mobile phase *n*-hexane:2-propanol:trifluoroacetic acid (80:20:0.1, *v/v/v*); flow rate 0.8 mL min^{-1}; detection on 250 nm.

3. Results and discussion

Three HPLC columns with various chiral selectors based on derivatized cyclofructans were used with normal phase eluents (different contents of *n*-hexane and 2-propanol or ethanol with small amount of trifluoroacetic acid) for the enantioseparation of spirobrassinin analogs. The reversal of enantiomer elution order for analytes was observed on cyclofructan 6 (CF6-RN, CF6-IP) and cyclofructan 7 (CF7-DMP) chiral columns (Fig. 1).

Next, the effect of column temperature in the range 10–40 °C on enantiomer elution order was examined. Based on the shape of elution curves of some analytes, the reversal of enantiomer elution order can be assumed. Unfortunately, we were not able to confirm the reversal of enantiomer elution order above calculated isoenantioselective temperature as it was out of allowed column temperature range. Further supporting experiments are in progress.

4. Conclusions

Reversal of enantiomer elution order of spirobrassinin derivatives induced by the type of cyclofructan chiral selector was observed in this study. The effect of temperature on the separation was also investigated. Temperature-induced reversal of enantiomer elution order was observed in some cases, but was not

proven because of the experimental limitations. Studies on these effects may provide valuable information for better understanding the mechanism of chiral recognition on cyclofructan chiral stationary phases and the optimization of separations.

Acknowledgments

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Separation of Enantiomers of Phenylalanine by HPLC Method With Different Types of Chiral Stationary Phases

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Keywords
chiral stationary phase
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Abstract
The present study is focused on HPLC separation of enantiomers of phenylalanine. Different types of chiral stationary phase (CSP) in normal-phase, reverse-phase, and polar-organic separation mode were used. The effect of stationary phase type and mobile phase composition was studied. CSPs based on teicoplanin and ristocetin in reverse-phase mode have the best enantioselectivity. Partial separation of enantiomers was achieved with stationary phase based on isopropylcarbamate cyclofructan six in polar-organic and normal--phase mode. CSPs based on β-cyclodextrin in polar-organic, normal--phase, or reverse-phase separation mode was not suitable for separation of enantiomers under study. The developed HPLC methods using CSPs with chiral selector teicoplanin and ristocetin were applied for determination of L- and D-phenylalanine in a dietary supplement and energy drinks. Limit of detection values for L- and D-phenylalanine were 0.05 and 0.06 $\mu g\,ml^{-1}$ (HPLC-UV method with teicoplanin CSP) and 0.13 and 0.14 $\mu g\,ml^{-1}$ (HPLC-UV method with ristocetin CSP), respectively.

1. Introduction

Enantiomers exhibit the same chemical and physical properties but have different biological activity. Because of that amino acids can have different behaviour in physical environment within chiral living organism. Therefore, the enantioseparations are important and indispensable in various fields of research and applications, mainly in pharmaceutical, food and environmental. In pharmaceutical industry, the examination of enantiomeric purity of drugs is important in view of pharmacokinetic and pharmacodynamics studies. Different enantiomeric forms of molecule can react with human body in different ways. Separation of amino acids enantiomers is important in food analysis because can provide information about quality control, processing, storage time effects, and contamination. Different enantiomenrs can give different taste (L-fenylalanin have sweet

taste and D-fenylalanin have bitter taste) or different aromas to foodstuff [1–3]. Several methods for enantioseparation of amino acids such as thin-layer chromatography, high-performance liquid chromatography, gas chromatography, capillary electrochtromatography, and electrophoresis were reported [3]. High-performance liquid chromatography (HPLC) has the higher application in the field of chiral analysis. Separation can be achieved indirectly based on formation diastereoizomeric complexes enantiomer-chiral derivatization reagent which can by separate on achiral column (e.g., C18). The other way is direct separation approach when diastereoizomers are formed on a strationary phase containing chiral selector (chiral stationary phase, CSP) or in mobile phase with addition of chiral selector [4].

This study is focused on direct separation of enantiomers of phenylalanine using CSPs based on β-cyclodextrin, isopropylcarbamate cyclofructan six (IP-CF6), teicoplanin, and ristocetin as chiral selectors. Effect of chiral stationary phase and composition of mobile phase were studied.

2. Experimental

2.1 Reagents and chemicals

D, L-phenylalanine (>98%) and L-phenylalanine (>98%) was purchased from Sigma Aldrich. Acetonitrile HPLC (VWR International, gradient grade purity), hexane (Sigma-Aldrich, for HPLC), ethanol (Centralchem, for UV), trifluoroacetic acid (Fisher Scientific, for HPLC), acetic acid (Centralchem, p.a.) and trimethylamine (Merck, for synthesis). Samples, dietary supplement, were obtained from a local pharmacy and energy drinks was bought from supermarket.

2.2 Instrumentation

The HPLC system consisted of degaser (Agilent Technologies 1260 Infinity), a delivery pump (Agilent Technologies 1260 Infinity), manual injector (Rheodyne) with a 20 µl sample loop, thermostat (Incos LCT 5100) and DAD detector (Agilent Technologies 1200) set at 210 nm, polarimetric detector (IBZ Messtechnik), circular dichroism based detector (Jasco). The chiral stationary phases based on teicoplanin (Chirobiotic T; 250×4 mm, 5 µm), ristocetin (Chirobiotic R; 250×4 mm, 5 µm), isopropylcarbamate cyclofructan 6 (IP-CF6; 250×4.6 mm, 5 µm) and β-cyclodexterin (Chiradex; 250×4.6 mm, 5 µm). The mobile phases were acetonitrile/water in reverse-phase mode, hexane/ethanol/trifluoroacetic acid in normal-phase mode and methanol/acetonitrile/acetic acid/triethylamine in polar-organic mode in different volume ratios. The flow rate was 0.8 ml min^{-1}.

Table 1
Comparison of retention factors (k), selectivity (α) and resolutions ($R_{i,j}$) of phenylalanine enantiomers for different types of chiral stationary phases in reverse-phase (RP), polar-organic (PO), and normal phase (NP) mode.

Stationary phase	β-cyclodextrin			IP-CF6		Teicoplanin	Ristocetin
Separation mode	RP[a]	PO[b]	NP[c]	PO[b]	NP[d]	RP[a]	RP[e]
k	2.82	1.68	4.19	0.96	5.96	0.95	1.17
α	1	1	1.22	1.07	1.39	1.33	1.42
$R_{i,j}$	0	0	1.07	0.54	2.20	1.59	3.32

Mobile phase: [a] acetonitrile/water (75/25, *v/v*), [b] methanol/acetonitrile/acetic acid/triethylamine (50/50/0.3/0.2, *v/v/v/v*), [c] hexane/ethanol/trifluoroacetic acid (75/25/0.2, *v/v/v*), [d] hexane/ethanol/trifluoroacetic acid (70/30/0.2, *v/v/v*), [e] acetonitrile/water (60/40, *v/v*).

3. Results and discussion

For the separation of enantiomers of phenylalanine different types of chiral selectors were tested. The enantioseparations were evaluated on the base values of retention factors and resolutions of enantiomers (Table 1).

CSP based on β-cyclodextrin was used in polar-organic, normal-phase and reverse-phase separation mode. In reverse-phase mode, mixture of acetonitrile and water was used as mobile phase. Amount of acetonitrile was ranged from 25 to 75%. In this separation mode enantiomeric forms were not separated. Mobile phase in polar-organic mode consist of methanol/acetonitrile/acetic acid/triethylamine. The methanol content in mobile phase varied from 25 to 75% and amount of ionic modifiers (0.3% acetic acid and 0.2% triethylamine) were constant. As in the reverse-phase mode, the separation of enantiomers was not achieved in polar-organic mode. This type of CSP was also tested in normal-phase separation mode. The hexane content in mobile phase containing hexane/-ethanol/trifluoroacetic acid varied from 25 to 75% and amount of trifluoacetic acid was not changed, 0.2%. Separation of enantiomers was achieved in mobile phase when hexane/ethanol/trifluoroacetic acid ratio was 75/25/0.2 (*v/v/v*). Obtained value of resolution was $R_{i,j} = 1.07$.

Chiral stationary phase based on IP-CF6 was tested in normal-phase and polar--organic mode. Both of these separation modes not provided separation of the enantiomers. In polar-organic mode mobile phase methanol/acetonitrile/acetic acid/triethylamine was used. Amount of methanol varied from 50 to 85% and amount of acetic acid was 0.3% and triethylamine was 0.2%. Increase of methanol content in mobile phase results in decrease of values of retention factor and resolution. The best mobile phase for separation of enantiomers of phenylalanine was methanol/acetonitrile/acetic acid/trimethylamine at ratio 50/50/0.3/0.2 (*v/v/v/v*), where the resolution value $R_{i,j} = 0.54$ was achieved. Higher efficiency of

enantioseparation on this CSP was obtained in normal-phase mode with mobile phase hexane/ethanol/trifluoroacetic acid. Amount of hexane varied from 50 to 70% and amount of trifluoretic acid was 0.1%. The best separation was achieved with mobile phase composition hexane/ethanol/trifluoroacetic acid 70/30/0.1 (*v/v/v*), where $R_{i,j} = 2.20$.

CSP based on macrocyclic antibiotics, teicoplanin and ristocetin, were used for separation of enantiomers of phenylalanine in reverse-phase separation mode. Mobile phase consisted of acetonitrile and water. For separation on CSP based on teicoplanin amount of acetonitrile in mobile phase varied from 30 to 90%. It was discovered that dependences of retention factors of enantiomers on composition of mobile phase was U-shaped. Values of retention factor decreased in interval from 30 to 45% and in the interval from 45 to 90% increased. The same trend was shown for resolution value. The suitable mobile phase composition for separation of enantiomers was acetonitrile/water (75/25, *v/v*), $R_{i,j} = 1.59$. For separation of phenylalanine enantiomers on CSP based on ristocetin were tested mobile phases containing acetonitrile and water in ratios from 30/75 to 85/15 (vol %). With increasing of content of acetonitrile in mobile phase, retention factors increased form 0.33 to 6.75 for L-phenylalanine and resolution increased from 2.48 to 4.11. The optimal mobile phase composition was acetonitrile/water (60/40, *v/v*) where resolution was $R_{i,j} = 3.32$.

Elution order of phenylalanine enantiomers was the same for all CSPs and mobile phases tested (retention factor value of D-phenylalanine was higher than for L-enantiomer).

These results show that the best separation of enantiomers of phenylalanine was achieved using CSPs based on teicoplanin and ristocetin. HPLC-UV methods were evaluated in view of limit of detection, limit of quantification, linearity and precision (Table 2). Polarimetric detector and circular dichroism detector were

Table 2
Sensitivity and precision of developed HPLC methods for determiantion of enantiomers of phenylalanine.

Chiral stationary phase		Teicoplanin[a]		Ristocetin[b]	
		L-phenylalanie	D-phenylalanine	L-phenylalanie	D-phenylalanine
LOD [μg ml^{-1}]		0.05	0.06	0.14	0.13
LOQ [μg ml^{-1}]		0.15	0.20	0.45	0.43
Linearity [μg ml^{-1}]		0.075–2.5	0.075–2.5	0.05–2.5	0.05–2.5
		2.5–500	2.5–500	2.5–250	2.5–250
R^2		0.991	0.999	0.992	0.999
Repeability in one day					
RSD [%]	t_R	0	0.2	0.1	0.1
	A	0.6	0.9	0.5	0.8
Repeability in six days					
RSD [%]	t_R	1.7	1.4	0.2	0.4
	A	3.5	1.8	4.5	3.3

Mobile phase: [a] acetonitrile/water (75/25, *v/v*), [b] acetonitrile/water (60/40, *v/v*).

also used for detection of phenylalanine enantiomers. These types of detectors were approximately 100-times less sensitive than spectrophotometric detector (at 210 nm).

Developed enantioselective HPLC method with teicoplanin and ristocetin CSPs were applied for analyses of real samples. Samples of dietary supplements contain both of enantiomeric forms and there were not any interference that could influenced the separation. In energy drink samples only L-enantiomer was detected at concentration levels higher than limit of detection. The recoveries of HPLC-UV method, determined at three concentration levels (from 21 to 357 mg D-, L-phenylalanine per 1 g of dietary supplements; from 1 to 5 mg of D-, L-phenylalanine to 1 liter of energy drink samples) were higher than 90 %.

4. Conclusions

This study compared chromatofraphic characteristics for separation of enantiomers of phenylalanine obtained on four different types of CSP. CSP based on β--cyclodextrin was not suitable for separation of enantiomers of phenylalanine in any tested mode. On IP-CF6 CSP was achieved enantioseparation in both tested modes, polar-organic, and normal-phase. Values of resolution were higher in normal-phase mode than in polar-organic mode. The best enantioselectivity was achieved using both of CSPs based on macrocyclic antiobiotics, teicoplanin, and ristocetin, in reversed-phase separation mode. Developed HPLC-UV methods were used for analyses of real samples. It was found that dietary supplement sample contain both of enantiomeric forms and energy drinks contain only L-enantiomer. Amount of D-enantiomer was below limit of detection of proposed method.

Acknowledgments

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Influence of pH on Retention Behavior of Polyaniline-Coated Silica Gel Stationary Phase Investigated Using Linear Solvation Energy Relationships in Capillary Liquid Chromatography

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Keywords
linear solvation energy relationships
polyaniline-coated silica gel
retention mechanism

Abstract
The retention behavior of polyaniline-coated silica gel stationary phase was investigated under the varied mobile phase composition utilizing the linear solvation energy relationships approach in capillary liquid chromatography. Almost 80 solutes of various chemistries were employed to identify dominant retention interactions, to propose a possibly multi-modal retention mechanism and to describe the effect of a substantial change of eluent buffer pH.

1. Introduction

Understanding the interactions occurring between a solute and a chromatographic system is crucial not only for the development of new separation methods, but also for method transfer from one column to another. Very popular simple chromatographic tests, e.g., Walters', Engelhardt's or Tanaka's, present easy-to--perform experimental approaches providing basic information, i.e., hydrophobicity, silanol activity, steric selectivity, etc., on the examined separation system [1]. These tests in combination with a subsequent chemometric treatment, i.e. employment of principal component analysis or hierarchical cluster analysis, allow mutual comparison or classification of chromatographic columns of similar chemistries. The simple tests can also be used for prediction of the suitability of a certain column for a required separation. However, these approaches cannot in principle provide any information on changes in retention interactions caused by changing measurement conditions, e.g., a mobile phase (MP) composition.

Fortunately, there also exists the linear solvation energy relationships (LSER) model, which relates thermodynamics of the retention process with interactions taking place between solutes and the separation system [2]. Extended Abraham's equation describes the relation between the overall analyte retention, expressed as retention factor k, and individual retention contributions

$$\log k = eE + sS + aA + bB + vV + d^{-}D^{-} + d^{+}D^{+} + c \quad (1)$$

where the capital letters are the solute descriptors describing certain structural characteristics of the solute in the form of a numerical value, the lower case letters are the system – stationary phase (SP) and MP – coefficients reflecting the particular type of interaction; e corresponds to the lone electron pair interactions and π-π stacking, s the dipole-dipole interactions, a the H-bond basicity, b the H-bond acidity, v the dispersion interactions (hydrophobicity), d^{-} the anion-exchange interaction, d^{+} the cation-exchange interaction and term c is the intercept, which is sometimes explained as the MP/SP ratio or retention interactions which are not included in the model (e.g., steric selectivity or SP throughput) [3]. The equation is solved out using multiple linear regression and obtained system coefficients are evaluated. The negative value of a coefficient refers to prevalence of an individual interaction between a solute and MP, said in other words, the solute is not retained on SP; the positive coefficient evinces for the opposite. The higher the absolute coefficient value, the more significant an interaction is. As the LSER system coefficients are dependent on temperature and composition of the MP, it is possible to evaluate the change of the retention interactions when the composition of eluent is modified [4].

Polyaniline (PANI) is a semi-conductive polymer of interesting structure (convertible based on conditions such as pH or applied potential) that can be readily synthesized in situ on various substrates and thus used as a surface modifier [5]. In this work we studied the retention behavior of PANI-coated silica gel (PANI-SiO_2) particles by employing LSER approach for two substantially different pH values of the MP buffer with respect to the protonation of amino and imino functionalities in PANI structure (Fig. 1).

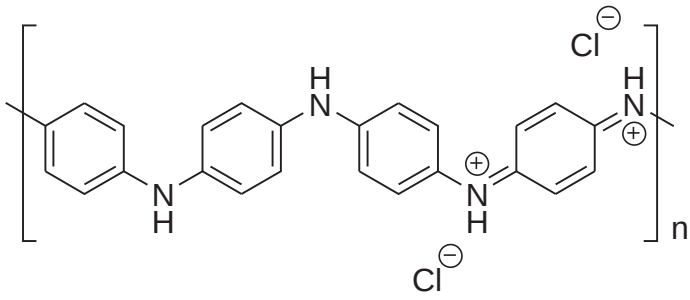

Fig. 1 Chemical structure of partly protonated polyaniline – emeraldine salt.

2. Experimental

2.1 Reagents and chemicals

Methanol (HPLC gradient grade) and solutes used for the LSER measurements (all of analytical grade purity) were purchased from Sigma–Aldrich (USA). Phosphoric acid and sodium hydroxide were supplied by Lachner (CZ). The deionized water was purified with a Milli-Q water purification system from Millipore (USA). Analyte solutions were prepared in a concentration range of 0.05–1.0 mg/mL either in CH_3OH/H_2O (50/50, *v/v*) or CH_3OH/100 mM sodium phosphate buffer (${}^{w}_{w}$pH = 2.5, ${}^{s}_{w}$pH = 3.4) depending on their solubility. The MPs used for retention measurements composed of 50/50 (*v/v*) CH_3OH/100 mM sodium phosphate buffer (${}^{w}_{w}$pH = 2.5, ${}^{s}_{w}$pH = 3.4) or 50/50 (*v/v*) CH_3OH/100 mM sodium phosphate buffer (${}^{w}_{w}$pH = 7.0, ${}^{s}_{w}$pH = 8.0). The aqueous ${}^{w}_{w}$pH values of the buffer solutions were adjusted before the addition of CH_3OH with concentrated H_3PO_4 or 10 M NaOH. Then the hydro-organic ${}^{s}_{w}$pH values of the complete MPs were measured, with the pH-meter calibrated in aqueous buffers.

2.2 Instrumentation

PANI-SiO_2 stationary phase was prepared by in situ chemical polymerization of aniline.HCl utilizing $(NH_4)_2S_2O_8$ as a reaction initiator. Detailed description of the sorbent preparation and the capillary column slurry packing procedure can be found in reference [6]. The thickness of PANI layer on the silica gel substrate is approximately 0.2 µm (measured using transmission electron microscopy).

Chromatographic measurements were performed using an Agilent 1200 Capillary LC System (Agilent Technologies, Germany) consisting of a vacuum degasser, binary pump, automated injector, column heating compartment and diode array detector. The ^{3D}HPLC ChemStation Software (Agilent Technologies) was used for acquisition and analysis of the experimental data. The injection volume was 0.1 µL, flowrate 5 µL/min, the capillary column was thermostated at 25 °C. UV detection was accomplished at 230 nm and 254 nm. The dead time was determined using a system peak. The ${}^{s}_{w}$pH values of the MP were used preferably for the calculations of D^- and D^+ descriptors since they are assumed to correspond more closely to the real ${}^{s}_{s}$pH value of the 50/50 (*v/v*) buffer-organic MPs than ${}^{w}_{w}$pH values of purely aqueous buffers [3, 7].

3. Results and discussion

The LSER model was established by using 79 solutes of various chemistries such as small organic bases, carboxylic acids, zwitterions, polar and non-polar neutral compounds, poly-aromatic hydrocarbons and even some positional isomers. The experimental log *k* values for the set of 75 solutes (only four outliers excluded for

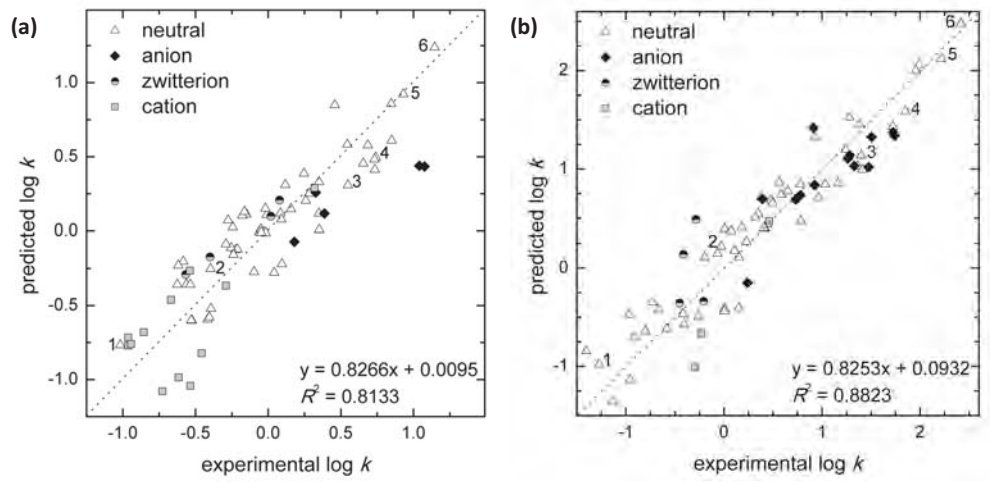

Fig. 2 Plot of experimental vs. predicted log *k* for different ${}^{s}_{w}$pH values of the mobile phases: (a) ${}^{s}_{w}$pH value of 3.4, (b) ${}^{s}_{w}$pH value of 8.0 (solutes are considered to be ions if at least 10% dissociated), the dotted line denotes equal log *k* values. Solutes: (1) acetone, (2) benzene, (3) naphthalene, (4) biphenyl, (5) anthracene, (6) pyrene.

each of MPs) show linear correlation with the calculated log *k* values with determination coefficients R^2 of 0.81 and 0.88 for acidic and mildly alkaline MP, respectively (Fig. 2a, b). From the perspective of LSER this means good agreement with a proposed model [1]. The slope values somewhat lower than unity indicate that solutes are slightly more retained than it is predicted by the model. Nevertheless, the shown graphs testify that solutes are equally scattered along the dotted line denoting equal log *k* values in both cases. It is worth noting that for the alkaline MP the log *k* span is almost doubled compared to the log *k* of the acidic MP. From general comparison of graphs of these two MPs of different ${}^{s}_{w}$pH values, the following is evident:

- cations are poorly retained under acidic MP conditions (except for 4-aminobenzoic acid) that is given by their Coulombic repulsion with protonated *N* atoms in PANI structure
- zwitterions are situated between cations and anions
- interestingly, anions interact with the SP more strongly under alkaline MP conditions
- polar neutral solutes are less retained than non-polar neutral solutes; it can be clearly seen that the retention of a solute increases with the number of aromatic rings in its structure (the same trend was observed by Chriswanto [8])
- presence of poorly retained neutral solutes in Fig. 2b) is given by the transformation of original cations into neutral compounds under slightly alkaline pH conditions.

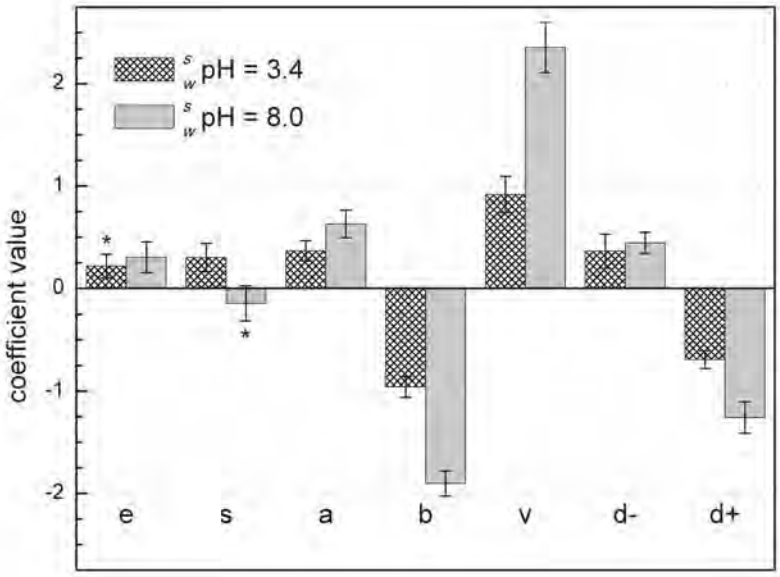

Fig. 3 System coefficients for different $^{s}_{w}$pH values of the eluent, provided with error bars denoting coefficient standard error and asterisks marking statistical insignificance of the coefficients.

According to the obtained system coefficients from LSER model, the *e* coefficient is statistically insignificant for explaining retention in the acidic MP (Fig. 3). It means that neither π-π nor non-binding electron pair interactions participate in the retention mechanism in this chromatographic system. Another explanation is that *e*-type interactions are of the same magnitude between the solute and the SP as between the solute and the MP, which is surprising with respect to the PANI structure composed of alternating benzene, benzenediamine and quinonediimine fragments. For the alkaline eluent *e* acquires statistical significance and mildly contributes to the overall solute retention on the SP. Dipole-dipole interactions between solute and SP, denoted as *s*, also slightly support the solute retention, although this applies only for acidic MP. For alkaline MP conditions, this coefficient is not considered as statistically significant. A positive value of *a* (H-bond basicity) reflects the ability of the SP to interact with acids; this trend even increases with rising pH value of the MP (as PANI is subjected to deprotonation). An expressive negative value of *b* (H-bond acidity) clearly corresponds to the acidity of phosphate buffer used and explains minute retention of cations (i.e. organic bases) on the SP. Growth of this coefficient under alkaline MP conditions expresses the difference in acidity of PANI and MP phosphate moieties, which can in contrast to PANI loose other protons. Significant hydrophobicity (*v*) of the SP compared to MP, that contains 50% of CH_3OH, causes considerable retention of non-polar neutral compounds. This phenomenon even magnifies under alkaline MP conditions. Apparent ability of the SP to act as a weak anion-exchanger, characterized

by d^-, could be attributed to residual protonated N atoms in the PANI structure or to the fact that SP can attract anions rather than constituents of the MP: phosphate and CH_3OH. The negative value of d^+ coefficient supports the above mentioned, cations would interact more preferably with partly dissociated phosphate moieties (${}^{w}_{w}\mathrm{p}K_{\mathrm{a},1} = 2.16$, ${}^{w}_{w}\mathrm{p}K_{\mathrm{a},1} = 7.21$) than with PANI containing (possibly still protonated) N atoms. Increase of the coefficient under alkaline MP conditions promotes that assumption. The relatively high system intercept c of values –1.06 and –1.31 for acidic and slightly alkaline MPs, respectively, suggest that steric throughput of PANI-SiO_2 stationary phase, definitely different from commercial single-layer SPs, could have certain retention-related effect as well. Generally we can say that by mere changing of MP buffer pH value, it is possible to substantially affect the retention behavior of PANI-SiO_2 stationary phase.

4. Conclusions

Although LSER model was not primarily intended for description of stationary phase retention behavior under modified mobile phase conditions, it was successfully applied for characterization of retention interactions of polyaniline-coated silica gel under varied pH of the eluent buffer. The dominant suggestible interactions of the system are hydrophobicity, H-bond acidity and basicity, and cation exchange. Therefore, this stationary phase offers multi-modal retention mechanism.

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Identification of Cyclitols in Morphological Alfalfa Parts Using Matrix-Assisted Laser Desorption Ionization With Time-of-Flight

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Keywords
alfalfa (*Medicago sativa L.*)
cyclitols
MALDI-TOF
mass spectrometry

Abstract
The study demonstrates the efficiency of Matrix Assisted Laser Desorption Ionization (MALDI) mass spectrometry in global analysis of cyclitols. Three cyclitols (D-pinitol, D-*chiro*-inositol, and L-*chiro*-inositol) were successfully identified in alfalfa extract (leaves, stem, and flowers) using MALDI-TOF MS in positive mode.

1. Introduction

Alfalfa (*Medicago sativa L.*) is important specie that has been used as feed for livestock due to its richness in proteins and vitamins [1]. Their content of bioactive components such as saponins, flavonoids, and cyclitols make from long decades a suitable plant for using in folk traditional medicine [2–4].

Cyclitols are a class of compound belong to sugar alcohol, widely distributed in plant kingdom and other living cells. Several studies showed involvement of inositols in various biological processes including cellular signalling, phosphate storage and their high efficiency as insulin-like properties enhancer for reducing blood glucose of human body [5, 6]. In addition, several studied demonstrated the biological activity of these compound as anti-oxidant, anti-inflammatory and anti-cancer agents [7–9].

2. Experimental

2.1 Reagents and chemicals

Cyclitols standards, D-pinitol (95% mass), L-*chiro*-inositol (95% mass) and D-*chiro*-inositol (≥ 98% mass) were purchased from Sigma-Aldrich (USA).

Methanol (for HPLC grade, ≥99.9) was purchased from Sigma–Aldrich (Germany). Ethanol (96%) was used for extraction procedure were obtained from Avantor (Poland). Ultra-pure water was obtained from a Milli-Q water system (Millipore, USA). Trifluoroacetic acid 10% was from Sigma-Aldrich (Germany) and acetonitrile was obtained from Polskie Odczynniki Chemiczne (Poland). MALDI matrix, α-cyano-4-hydroxycinnamic acid was obtained from Fluka Feinchemikalien (Germany). Trimethylsilylimidazole was used for derivatization step and purchased from Sigma-Aldrich (USA).

2.2 Instrumentation

Mass spectrometric analyses were performed using an ultra-feXtreme MALDI--TOF/TOF mass spectrometer (Bruker Daltonics, Germany). The system equipped with a modified neodymium-doped yttrium aluminium garnet (Nd:YAG) laser (smartbeam II) operating at the wavelength of 355 nm and a frequency of 2 kHz. All spectra were recorded in reflector positive mode using acceleration voltage of 25 kV within *m/z* range of 100–1000 Da at 90% of laser power and global attenuator 50%.

GC–MS analyses were carried out in an AutoSystem XL gas chromatograph coupled to TurboMass mass spectrometer in the splitless mode (both from Perkin Elmer, USA) using He at 1 mL min^{-1} as carrier gas. A RTX-5MS capillary column 30 m × 0.25 mm, 0.10 μm thickness by (Restek, USA) was used.

3. Results and discussion

Samples were extracted and purified using Soxhlet extraction method and solid-phase extraction. We successfully identified the presence of three different cyclitols: D-*chiro*-inositol, L-*chiro*-inositol, and D-pinitol in different parts (leaves, stem, and flowers) of alfalfa extracts using MALDI-TOF MS in positive-mode. The same cyclitols were identified and quantified using GC-MS. The results of MALDI analysis highlighted that all investigated parts of alfalfa plant contain cyclitols, but the stem seems to be more abundant than other parts. The same results were obtained by GC-MS analysis and proved that the stem extract contained the highest quantities of cyclitol than the other parts. All ions fragments associated with L-*chiro*-inositol were found in stem extract. D-*chiro*-inositol fragments were prevalent in both leaves and stem. Ion fragments coming from D-pinitol were predominant in all leaves extract. Fewer fragments of cyclitols were found in flowers compared with leaves and stem. Figure 1 (on next page) show the distribution of peaks between α-cyano-4-hydroxycinnamic acid matrix and alfalfa extracts. While, Fig. 2 shows the cyclitols present in the leaves of alfalfa extract.

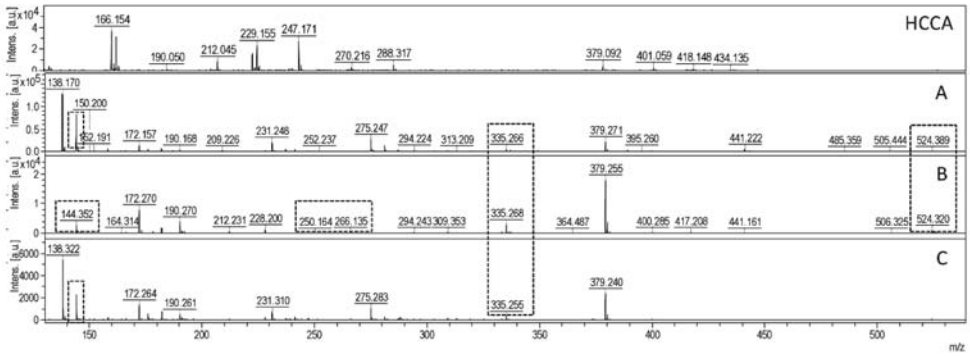

Fig. 1 Signals distribution between α-cyano-4-hydroxycinnamic acid matrix and alfalfa extracts, where: (A) leaves extract, (B) stem extract, and (C) flowers extract.

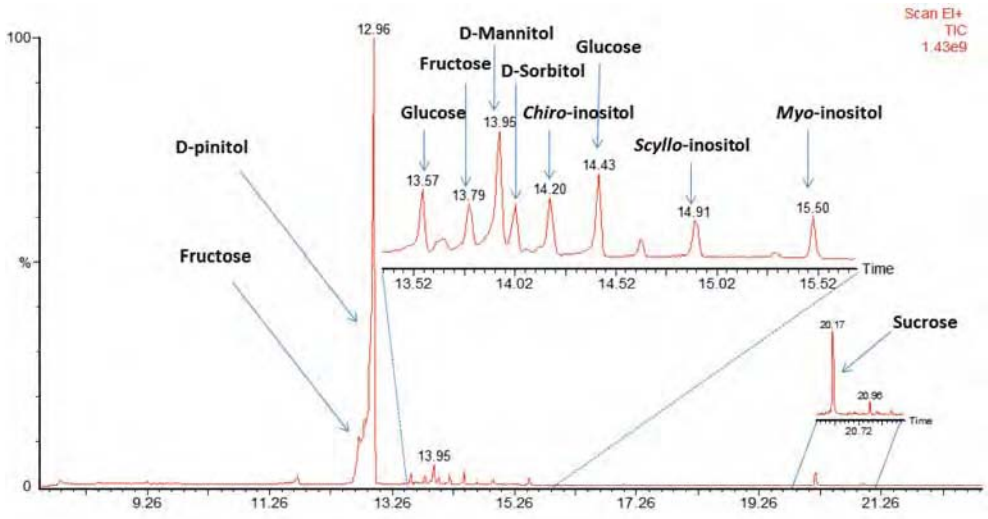

Fig. 2 GC-MS profile of leaves extract of alfalfa.

4. Conclusions

In this study, using MALDI-TOF MS as rapid and sensitive soft ionization technique, we successfully identified three cyclitols present in alfalfa extract (leaves, stem, and flowers). In addition, we successfully demonstrated the potential of MALDI--TOF mass spectrometry for cyclitols identification and the efficiency of this technique to differentiate between cyclitols isomers. The GC-MS analysis was a confirmation of MALDI analysis.

Acknowledgments

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Voltammetric Techniques for Analysis in a Single Drop of a Solution

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Keywords
glassy carbon electrode
miniaturization
voltammetry

Abstract
This contribution describes miniaturization of voltammetric methods and some of the main problems caused by reducing the sample volume to 20 μL. This study was carried out in dimethyl sulfoxide solutions and buffered aqueous solutions with 10% DMSO at a glassy carbon electrode. A novel antibiotic agent, 1-hydroxy-*N*-(4-nitrophenyl)naphthalene-2-carboxamide, was used as a model substance. This analyte was determined by cathodic and anodic voltammetry. Elimination of the negative influence of dissolved oxygen was performed in various manners. Two most effective methods were square wave voltammetry that can be used in the presence of dissolved oxygen and removal of oxygen in a microcell with nitrogen atmosphere inside.

1. Introduction

Electrochemical micro systems have a great importance in analysis of biochemical systems because of its simplicity, rapidity, selectivity, and low sample and reagent consumption [1] . They are especially favourable in analysis of biological or biochemical samples that usually have limited volume ranging from tenths to hundreds of microliters. Therefore, it is necessary to adjust voltammetric techniques accordingly and to use miniaturized voltammetric cells. These microcells can be used as flow-through detectors coupled with chromatographic or flow injection analysis or they can work in batch arrangements with static samples introduced into the system by a pipette or other similar devices. There are two basic configurations of the working electrode. It could be in its usual state set from top to bottom and immersed in a small sample volume or in a more

commonly used inverted state set from bottom to top that favours possibility of applying smaller volumes on its surface. The miniaturization also encourages the use of working microelectrodes [2]. Various simple electrochemical microcells have been introduced till now [3–5].

In our previous work we have introduced a method for the determination of 4-nitrophenol by cathodic voltammetry in dimethyl sulfoxide (DMSO) in various microcell designs [6] and reported about the voltammetric determination of 1-hydroxy-*N*-(4-nitrophenyl)naphthalene-2-carboxamide (HNN) in a single drop of a solution by anodic and cathodic voltammetry [7–9]. This compound is a novel antimycobacterial agent [10–12] and it is necessary to develop a method for the determination of these compounds in as small volume as possible because of their limited amount available. The aim of this contribution is to summarize our previous findings and add some new, recently obtained data.

2. Experimental

2.1 Reagents and chemicals

A stock solution of HNN (1-hydroxy-4'-nitro-2-naphthanilide; CAS number: 68352-27-2; Sigma Aldrich, Germany) was prepared by dissolving the substance in dimethyl sulfoxide (Penta, Czech Republic). Tetrabutylammonium tetrafluoroborate (p.a., Sigma Aldrich, Germany) was used as a supporting electrolyte in DMSO. Britton-Robinson buffer was prepared by mixing 0.04 mol L^{-1} phosphoric, boric, and acetic acid with 0.1 mol L^{-1} sodium hydroxide (all p.a. Lach-ner, Czech Republic). Bacterial growth medium contained 0.4 mol L^{-1} sucrose (white refined granulated sugar, Investice Strategie Management a.s., Czech Republic); 20 mmol L^{-1} phosphoric buffer (pH = 7.2) prepared by mixing disodium hydrogen phosphate and sodium dihydrogen phosphate (p.a. Lach-ner); 5 mmol L^{-1} sodium chloride (p.a. Lach-ner); and 15 mmol L^{-1} magnesium chloride (p.a. Lachema, Czech Republic).

2.2 Instrumentation

Voltammetric measurements were carried out in a three-electrode system with glassy carbon disc working electrode (GCE, 2 mm diameter, Metrohm, Switzerland), auxiliary platinum wire electrode (Eco-Trend Plus, Czech Republic) and a Ag|AgCl (3M KCl, Elektrochemicke detektory) reference electrode or a non-aqueous Ag|$AgNO_3$ (0.01 mol L^{-1}), Bu_4NBF_4 (0.1 mol L^{-1}) in DMSO reference electrode. Additionally, platinum (0.5 mm) and silver (0.4 mm) wires were used as auxiliary and pseudo-reference electrode, respectively. GCE was polished prior to measurements with aqueous slurry of alumina powder (1.1 μm) to mirror-like appearance. Differential pulse voltammetry (DPV) was carried out using scan rate 20 mV s^{-1}, pulse width –100 ms, and pulse amplitude –50 mV. Square wave

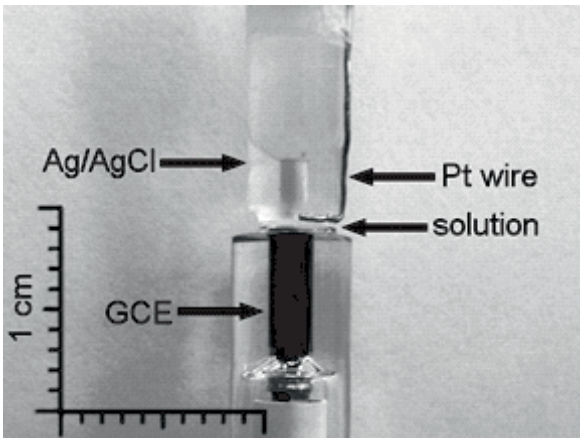

Fig. 1 Microcell for anodic voltammetry with GCE, Ag|AgCl reference electrode and platinum wire auxiliary electrode with 20 μL sample.

voltammetry (SWV) was carried out at frequency 100 Hz, pulse amplitude –50 mV, and step – 4 mV. 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). Electrode configuration and work with a drop was described in detail in [6]. After electrode assembly, 20 μL drop of a solution was carefully pipetted onto the working electrode. Configuration of miniaturized voltammetric cell for anodic DPV is photographed at Fig. 1.

3. Results and discussion

This study was carried out by methods of anodic and also cathodic voltammetry in dimethyl sulfoxide (DMSO) solutions or aqueous buffered solutions usually containing 10% of DMSO. Cathodic voltammetry had one obvious problem and that was the cathodic oxygen response. In the case of 4-nitrophenol [6] and HNN [8] SWV in DMSO proved to be a fast way to determine these compounds. After optimization of this method it was possible to determine these compounds in DMSO solutions with the dissolved oxygen. Signals of oxygen and analytes were influenced by fast square wave voltammetric scans in a different way because their response was influenced by the reversibility of the corresponding processes. However, as we moved to aqueous solutions, SWV did not work as anticipated [9]. Other ways of removing oxygen, such as by the well-known reaction with sodium sulphite also caused the reduction of the analyte that was observed as a rapid decrease of peak height and the presence of the reduction product in the solution detected in a first voltammetric scan. Therefore, it was necessary to remove oxygen in a configuration described in [6]. This process takes much longer time than in a commonly used 10 mL sample. The drop is not stirred and dissolved oxygen is presumably only removed from the surface of a drop and it has to be transported there from the middle of a drop by the slow diffusion process. This process can take up to 20 minutes. However, this caused other problems especially

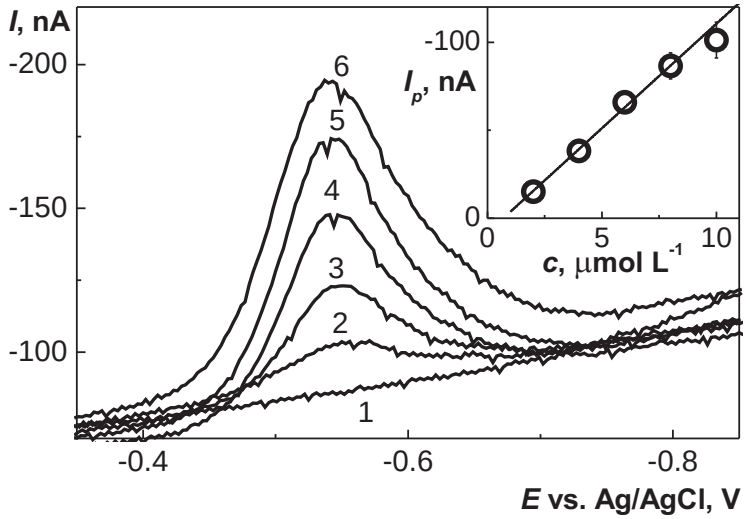

Fig. 2 Cathodic DP voltammograms of HNN of concentration (1) 0.0, (2) 2.0, (3) 4.0, (4) 6.0, (5) 8.0, and (6) 10.0 μmol L^{-1} obtained at GCE in one drop (20 μL) of BR buffer (pH = 7) and DMSO (9:1) solution after 10 min in the nitrogen atmosphere measured against silver wire pseudo-reference electrode (adjusted for Ag|AgCl in the figure). The calibration curve is shown in the inset.

in aqueous solutions because during that time the analyte from the drop was transported by diffusion to the reference electrode filling solution or it got adsorbed on the frit. Silver wire pseudo-reference electrode was then used. However, this can be only acceptable in analytical applications where it is not necessary to precisely know the peak potential as the potential of this pseudo-reference electrode was not stable. This also influenced the removal of the oxygen because the drop has a larger surface exposed in the nitrogen atmosphere resulting in the more effective oxygen removal and shortened time down to 10 minutes. However, DPV exhibited better sensitivity than SWV in determination of HNN in the solution without dissolved oxygen.

When working with small samples it is necessary to keep in mind the effect of electrolysis on a concentration in a drop. Usually in 10 mL the decrease of concentration in a bulk resulting from one voltammetric scan is negligible. In a 20 μL drop one scan can result in a significant drop of concentration around 10% (or more) with the same electrode (0.2 mm diameter) especially when working with concentrations near the limit of quantification. Because of that, a new drop had to be pipetted before every measurement. However, at higher concentrations the signal did not decrease significantly after the first scan and was stable in consequent scans. It was possible to measure one calibration point (5 scans) in one drop (Fig. 2). Another reason for applying new drop before every measurement was electrode fouling observed during anodic DPV of HNN even in 10 mL.

Applicability of the method was evaluated in a real matrix, the bacterial growth medium [9]. In this case the removal of oxygen took longer, 15 minutes, with the silver pseudo-reference electrode presumably because of slower diffusion of oxygen in a concentrated sucrose solution. However, HNN peak splitting to a double-peak in some voltammetric scans was observed in this case. This phenomenon is still under investigation and so far unexplained. However, this problem can be circumvented by increasing the volume of the drop to 50 µL. This indicates some other potential problem with the proposed microcell which must be further investigated.

4. Conclusions

The voltammetric method for determination of a novel antibiotic agent, 1-hydroxy-*N*-(4-nitrophenyl)naphthalene-2-carboxamide, in a single drop of solution has been developed. Problems caused by miniaturization of a classic voltammetric cell were satisfactorily resolved.

Acknowledgments

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Enzymatic Reaction of α-Amylase With Starch and Its Monitoring by an Online Capillary Electrophoresis Method

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Keywords
α-amylase
capillary electrophoresis
online enzyme assays
starch

Abstract
Pancreatic α-amylase plays a crucial role in polysaccharide metabolism located in small intestine. Thus, inhibition of this enzyme is important for patients suffering from diabetes mellitus. In this study, we developed an online capillary electrophoresis method to monitor time course of enzymatic reaction of α-amylase with starch. Since the enzymatic reaction takes place in a capillary and the introduction process is automated by introducing zones of substrate and enzyme separately, fast screening of possible inhibitors is enabled within 6 minutes. Moreover, consumption of enzyme and substrate is decreased significantly.

1. Introduction

Diabetes mellitus belongs among the most serious civilization diseases with an estimated 422 million of patients worldwide in 2014 [1]. Inhibition of polysaccharide-degrading enzymes such as α-amylase and α-glucosidase is essential for diabetes patients. Commercially available inhibitor drugs (acarbose, voglibose) of microbial origin often tend to cause unpleasant side effects. Therefore, research in this field aims at naturally occurring compounds such as flavonoids as potential inhibitors over the last few years [2].

Historically, UV-VIS spectrophotometry has been the most widespread method for study of substrate-enzyme reactions [3] and thus for screening if inhibitors. As new electromigration techniques were developed, different possibilities for study of enzymatic reactions arose, e.g., electrophoretically mediated microanalysis (EMMA), which comprises introduction of enzyme and substrate zone as well as incubation of the mixture and separation of reaction products in a single run inside the separation capillary [4]. This technique was further improved with transverse diffusion of laminar flow profiles (TDLFP), which contributed to better

mixing of enzyme and substrate zones. The TDLFP technique is based on hydrodynamic introduction of a few enzyme zones between several zones of substrate. The zones introduced by pressure create plugs with longitudinal interfaces, subsequently they are mixed by transverse diffusion [5].

In this study, we developed an online capillary electrophoresis method to determine the degradation rate of starch in the presence of α-amylase. The same composition of background electrolyte as in already published study [6] was used; nevertheless, the method was further optimized to be suitable for online experiments.

2. Experimental

2.1 Reagents and chemicals

Soluble starch p.a. (Penta, Czech Republic) was used for the preliminary offline experiments as well as for the online measurements. α-amylase was prepared from Orenzym tablets (Noventis, Czech Republic), the tablets were deprived of the cover layer and the inner part was lyophilized. Sodium chloride p.a. and acetic acid p.a. (99%) were purchased from Lach-Ner (Czech Republic), iodine was purchased from Lachema (Czech Republic), sodium dihydrogenphosphate and sodium hydrogenphosphate were purchased from Merck (Germany). Sodium hydroxide was purchased from Penta (Czech Republic). Solutions were prepared using deionized water supplied by a Milli-Q water purification system from Millipore (USA).

2.2 Instrumentation

All electrophoretic experiments were conducted using a G7100A Capillary Electrophoresis Instrument (Agilent Technologies, Waldbronn, Germany) with UV-VIS detector operating at 560 nm wavelength.

Measurements were conducted in a fused-silica capillary (Polymicro Technologies, USA) of 75 μm ID and 363 μm OD with the total length of 70.0 cm and 8.5 cm to detector. The capillary was flushed with 1 M NaOH (10 min) followed by deionized water (10 min) before the first use. Prior to every analysis, the capillary was rinsed with 0.1 M NaOH (30 s) followed by deionized water (30 s) and background electrolyte (2 min). In offline measurements, the sample was introduced hydrodynamically by pressure of 5 kPa for 3 s. In online experiments, one zone of α-amylase was introduced (5 kPa × 1 s) between two zones of starch (5 kPa × 1 s) followed by additional zone of background electrolyte (5 kPa × 1s) at the detector end of the capillary and voltage of –20 kV was applied inducing an electric current of app. 34 μA. Background electrolyte was composed of 20 mM acetate buffer of pH = 5.0 (adjusted with 1 M NaOH) with addition of 1.2 mM iodine solution (50 mM iodine dissolved in 300 mM potassium iodide). Triiodide ions, present in

background electrolyte, bind to starch molecule creating blue colored complex absorbing at 560 nm. All the measurements were conducted at 37 °C. The reaction constituents such as α-amylase and sodium chloride were dissolved in 10 mM phosphate buffer of pH = 7.0.

3. Results and discussion

In both offline and online arrangement of experiments, poor repeatability of peak areas occurred. Therefore, thorough flushing procedure was prefixed before every analysis, which contributed to increase of peak area repeatability with *RSD* below 5%.

3.1 Offline experiments

Firstly, the ability of the capillary electrophoresis method to reliably monitor starch degradation process was tested in an offline arrangement of experiment. Since commonly available starch is poorly soluble in water, it was dissolved in 90% dimethyl sulfoxide to yield 1% *w/v* solution. The presence of chloride anions in an incubation medium is essential for proper function of α-amylase, therefore the incubation medium was prepared mixing starch, sodium chloride and α-amylase

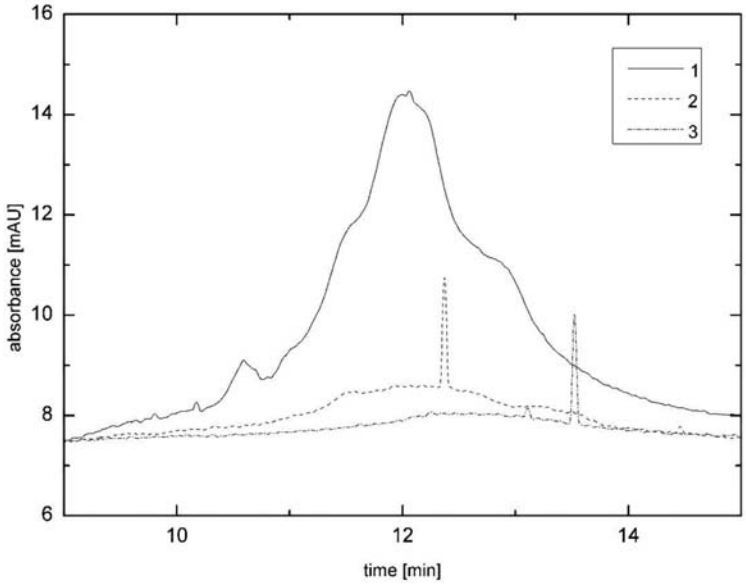

Fig. 1 Time course of starch degradation by α-amylase in offline mode. The initial concentration of starch was 0.1% *w/v*. Experiment was conducted in 10 mM phosphate buffer of pH = 7.0 at 37°C. Aliquots were measured after (1) 9.5 min, (2) 29.5 min, and (3) 52 min. Capillary electrophoresis conditions: 20 mM acetate buffer of pH = 5.0 with addition of 1.2 mM iodine as background electrolyte, U = 20 kV.

Fig. 2 Introduction of reaction zones into the capillary during online experiments. One zone of α-amylase (E) introduced between two zones of starch (S) with additional zone of background electrolyte (BGE) for better distribution of reaction medium.

solution with 10 mM phosphate buffer of pH = 7.0 to give the following constituent concentrations: 0.1% starch; 0.01 M chloride anions; 0.1 mg/ml α-amylase. The reaction mixture was kept at 37 °C and aliquots were removed and measured after certain periods of time. The aliquots were measured under the conditions of normal polarity mode capillary electrophoresis. As seen in Fig. 1, the starch peak area decreases with time rapidly, which confirmed suitability of the proposed method for online capillary electrophoresis experiments.

3.2 Online experiments

Four zones were introduced into the capillary in total, according to the scheme in Fig. 2 under the reverse polarity mode capillary electrophoresis (for sample introduction see section 2.2). The initial concentration of starch solution for online experiments was 0.08% *w/v* (the calibration curve for starch concentration between 0.01 and 0.08% was linear with R^2 = 0.9850) containing phosphate buffer (pH = 7.0) and 0.01 M sodium chloride. Concentration of α-amylase dissolved in 10 mM phosphate buffer was 0.1 mg/ml. After introduction of all the zones, the reaction medium was incubated for 0, 30, 60, 120, 300 and 600 s. The dependence of concentration on time (as seen in Fig. 3) was logarithmized (excluding the last point), which provided linear dependence of ln *c* on time. This fact supports the theory that degradation process of starch with α-amylase follows the first order reaction kinetics until app. 300 s. Then, presumably, the breakdown of starch molecules follows zero order reaction kinetics with linear decreasing course. As a comparison, there is Fig. 4 showing electropherograms after the longest (A) and shortest (B) incubation periods. There are two peaks indicating presence of amylopectin and amylose as proposed in the previous study [6]. However, these peaks were not totally resolved under the same conditions in our study.

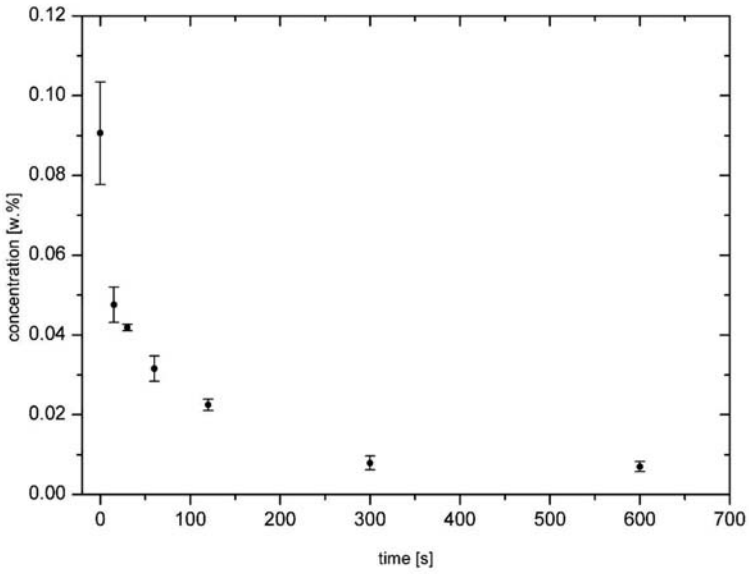

Fig. 3 Dependence of starch concentration on time in online arrangement. Initial starch concentration was 0.08% *w/v*, concentration of α-amylase was 0.1 mg/ml. Background electrolyte: 20 mM acetate buffer (pH = 5.0) with 1.2 mM iodine, $U = -20$ kV, hydrodynamic introduction (5 kPa × 1 s) of one zone of α-amylase between two zones of starch. Experiments conducted at 37 °C. Error bars represent the standard deviations of three measurements.

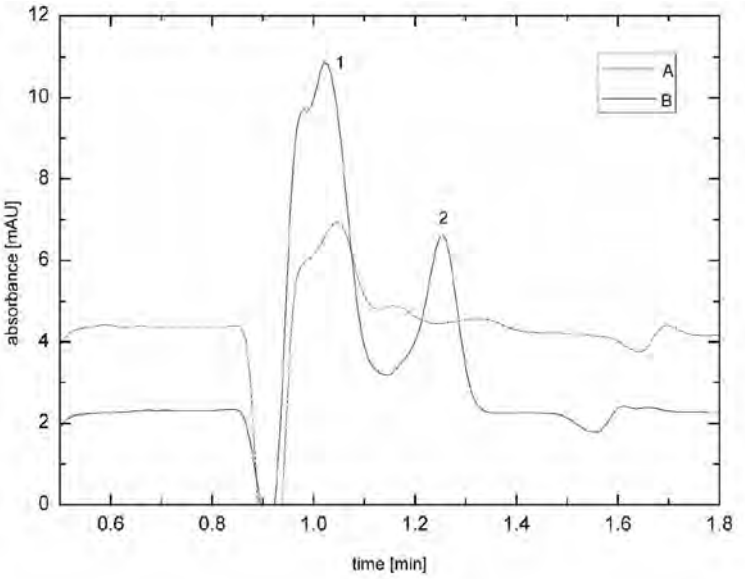

Fig. 4 Online time course of starch degradation by α-amylase. Capillary electrophoresis conditions same as in Fig. 1, measured in reversed polarity mode ($U = -20$ kV) at 37°C. Different incubation times: (A) 10 min, (B) 0.5 min. Peaks: (1) amylopectin, (2) amylose.

4. Conclusions

An online method for study of reaction kinetics of starch with α-amylase was developed. Normal polarity capillary electrophoresis did not provide satisfactory resolution and shape of the peaks. Therefore, the arrangement was changed and samples were introduced at the detector, shorter end of the capillary using reverse mode capillary electrophoresis, which improved peak shape and reduced analysis time. Preconditioning step, including capillary flushing, of the analysis is considered to have a major effect on repeatability of peak areas.

Acknowledgments

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Effect of Antioxidant Activity of Flavonoids and Phenolics Isolated by Supercritical Fluid Extraction from *Medicago sativa* Leaves

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Keywords
alfalfa (*Medicago sativa L.*)
supercritical fluids extraction
total phenols content
flavonoids
antioxidant activity

Abstract
The aim of study was to investigate the effect of supercritical fluid extraction (SFE) parameters including pressure, temperature, and ethanol concentration on the yield, content of major flavonoids, phenolics and antioxidant activity. The total concentration of flavonoids and phenolics was determined spectrophotometrically. Moreover, the concentration of selected compounds was determined chromatographically and their structures were confirmed by mass spectrometry. Antioxidant potential was measured spectrophotometrically with using DPPH and ABTS method. Preliminary single-factor experiments enabled selection of the optimal parameters such as type of co-solvent, time of extraction and flow rate. In the next step, the effect of temperature and pressure was studied. Results showed that total flavonoids and phenolics content as well as antioxidant activity measured with DPPH increase with temperature and pressure up to 70 °C and 300 bar, respectively. Above these parameters the decrease of the level of studied compounds was observed.

1.Introduction

Medicago sativa L. (lucerne or alfalfa) is widely grown throughout the world as a forage. Beside high protein content, this plant contains many valuable secondary metabolites as biological active compounds from the groups of phenols and flavonoids [1–3].

The conventional methods of extraction of plant material with organic solvents have many drawbacks, such as low selectivity or consumption of large volume of solvent. Nowadays, from the point of view of green chemistry, supercritical fluid extraction (SFE) has gained much popularity [4, 5]. This method belongs to environment-friendly technologies that represent an alternative to conventional extraction methods. SFE replace toxic organic solvent with benign fluid, such us

carbon dioxide. Due to the fact that carbon dioxide is non-polar, modification of SC-CO_2 through co-solvent or modifiers is needed to extract bioactive compounds [6].

The objective of this study was to investigate the effect of SFE extraction parameters including pressure, temperature and ethanol concentration on the yield, content of major flavonoids, phenolics and antioxidant activity.

2. Experimental

2.1 Chemicals and plant material

All chemicals and reagents were of analytical grade and were purchased from Sigma Aldrich, Germany.

Medicago sativa (varius alpha) leaves used in this study were harvested in National Research Institute, Kołuda Wielka, Poland. Alfalfa leaves were dried at the temperature of 50 °C for 48 h. The dried samples were milled to a fine powder using a laboratory mill. The obtained material was stored in the dark places until further use.

2.2 Extraction of the plant material

The maceration was performed using 250 mg sample which was soaked in 10 mL ethanol (96%, *v/v*) for 24 h at 50 °C in the dark. For ultrasound-assisted extraction (UAE) mixture was sonicated (2×30 min) and incubated for 24 h at 50 °C in the dark. The accelerated solvent extraction (ASE) was performed by using a Dionex ASE 350 System (USA). A sample (500 mg) was mixed with an amount of sand and the extraction was performed under the conditions: 96% ethanol as solvent, temperature 50 °C, pressure 10 MPa, static time 5 min, static cycle 2, purge time 100 s. The supercritical fluid extraction (SFE) was performed by using MV-10 ASFE Systems (USA). A sample (500 mg) was loaded into the SFE cell, then completed with glass bead. Extractions were carried out at 50°C, at a pressure of 10 MPa with a 60 min static period, and 10 min dynamic mode (continual flow) with using SC-CO_2 as solvent, and 96% ethanol as co-solvent. The flow rates were: SC-CO_2 at 4 mL/min, 96% EtOH at 1 mL/min.

2.3 Determination of total phenolic and total flavonoid content

The Folin-Ciocalteu method was used to determine the total phenolic content (TPC). The reaction mixture was prepared by mixing 0.1 mL of the extract, 1.5 mL deionized water, 0.1 mL Folin-Ciocalteu reagent, and 0.3 mL Na_2CO_3 (20%, *w/w*) (after 8 minutes). The absorbance was measured at 765 nm, after incubation at room temperature for 30 minutes in the dark. Gallic acid was used as a calibration standard.

The total flavonoid content (TFC) was measured according to aluminium chloride colorimetric assay [7]. The aliquots (0.5 mL) of extract were mixed with the same volume of 2% $AlCl_3$ in ethanol and then diluted with ethanol to 2 mL. The solution was mixed well and the absorbance was measured against prepared reagent blank at 420 nm, after 40 min at ambient temperature. Rutin was used as a calibration standard and the results were expressed as rutin equivalents (RE).

2.4 Determinations of antioxidant activity

The effect of the samples on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined using DPPH radical scavenging method. 1.2 mL of 0.1 mM ethanolic solution of DPPH was added to 0.3 mL of the extract. The sample absorbance was read at 517 nm after 30 min incubation. DPPH radical scavenging activity was expressed as mg of trolox equivalents (mg TEAC/g dry extract).

2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^{\bullet+}$) was generated in the reaction of 7.4 mM ABTS stock solution with 2.6 mM potassium persulfate. The mixture was left to stand in the dark at room temperature for 12 h before use. The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance at 734 nm of 0.70±0.02. 12 µL of the sample was added to 238 µL of diluted $ABTS^{\bullet+}$ solution, then the absorbance was measured after 2 h at 734 nm. $ABTS^{\bullet+}$ radical scavenging activity was expressed as mg of trolox equivalents (mg TEAC/g dry extract).

2.5 HPLC/MS investigation

The apparatus was a Shimadzu LC-MS 8050 system (Japan). Extract of leaves from *Medicago sativa* L. were separated using a phenyl-hexyl column (100×2.1 mm, 1.7 µm, Phenomenex, USA). The flow rate was 0.2 mL/min, injection volume was 20 µL, column temperature was 25 °C, the mobile phase gradient was 0 min: 10% B + 90% A, 3 min: 10% B + 90% A, 13 min: 80% B +20% A, where A 0.1% formic acid in water, B was acetonitrile.

3. Results and discussion

Yield of extraction processes is correlated with type of solvent, its polarity, pH, temperature, extraction time, and composition of the sample. Studies showed that the most efficient method of extraction for leaves was ASE with 70% ethanol (Fig. 1a, next page). Extraction with SC-CO_2 with ethanol as co-solvent gave lower efficiency than other tested extraction techniques. In our study all the techniques of isolation with 96% ethanol were found to achieve lower yields in comparison to correlated techniques with 70% ethanol, which suggests that compounds other than phenolics may have been extracted by aqueous ethanol (70%) and contribute to higher yield.

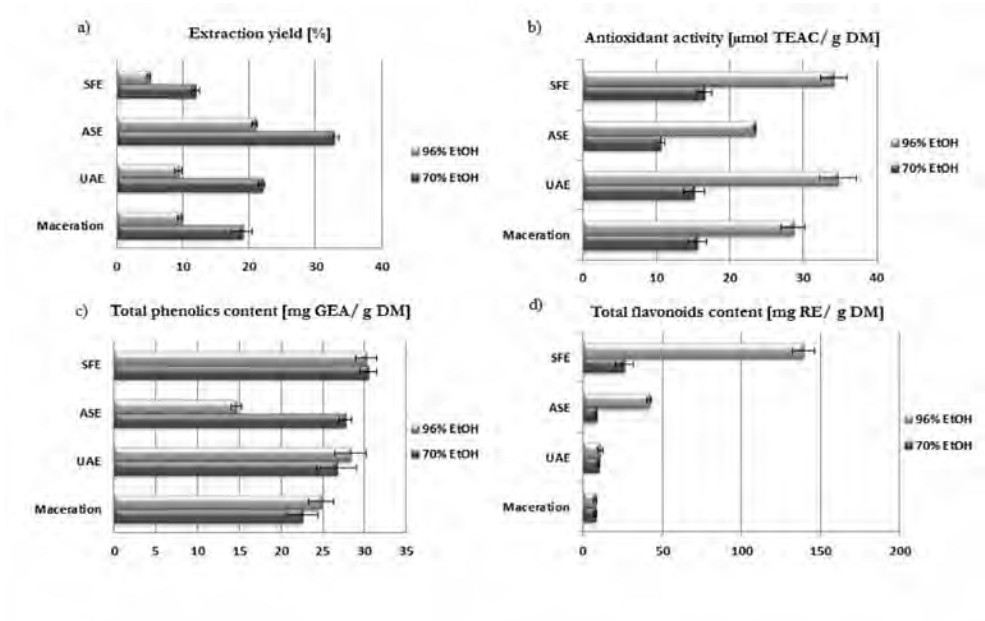

Fig. 1 (a) Extraction yields, (b) antioxidant potential, (c) level of total phenolics content, and (d) total flavonoids content in *Medicago sativa* leaves extracts obtained with using different techniques of extraction.

The obtained results showed the influence of the used extraction methods and concentration of solvent on the observed antioxidant activity (Fig. 1b). In our case, extracts obtained with using 96% ethanol were more effective than 70% ethanol. The high antioxidant activity was observed for extracts obtained with SFE and UAE. Moreover, studies showed that despite the low yield of SFE, the obtained extracts are characterized by high content of compounds having antioxidant properties. Despite the fact that SFE gave the lowest yields, the level of TPC and TFC in these extracts was very high (Fig. 1c, d).

Due to the fact that the SFE is a fast, selective and environmentally friendly extraction method, as well as preliminary results for extraction of *M. sativa* were promising, we carried out the initial selection of SFE parameters. In the first step the effect of added modifier on quality of extraction was tested. Two different solvent concentrations were used as a modifier, i.e. 70% and 96% (*v/v*) ethanol. Although the extraction efficiency for 70% ethanol was significantly better, the total content of polyphenols practically did not differ, but both flavonoid content and total antioxidant capacity were significantly higher for 96% ethanol as a modifier. Therefore, 96% ethanol was selected for further studies.

In the next step the effect of extraction time on polyphenol content and antioxidant activity was tested. SFE could be carried out statically and dynamically or combining both types. Samples with a compact matrix structure, containing hardly soluble analytes, must have provided long contact with the extractant

Table 1
Comparison of extraction yield, level of total phenolic (TPC) and flavonoid (TFC) content, and antioxidant potential (DPPH) of extracts obtained with using different SFE conditions.

Condition	Extraction yield [%]	TPC [mg GAE/ g DM]	TFC [mg RE/ g DM]	DPPH [μmol TEAC/ g DM]
Co-solvent				
96%	4.9±0.2	30.2±1.2	139.0±7.1	34.1±1.8
70%	11.9±0.6	30.4±1.0	25.8±5.7	16.5±1.0
Static time				
15 min	2.3±0.4	27.5±7.7	97.5±18.5	31.3±2.6
30 min	3.0±0.4	29.5±8.5	109.8±12.8	31.1±0.1
60 min	3.8±0.2	30.5±4.8	135.9±10.6	33.5±0.3

(static method). The dynamic method is used for samples readily penetrated by liquid and containing analytes well soluble in it.

Under the same extraction conditions as previously, three extraction times were compared (Table 1). In all cases, the dynamic time was 10 minutes, respectively. There were no significant differences in the results, so the optimum time, i.e. 30 minutes of static mode, was selected for further determinations.

Once established conditions: extraction time 40 min, flow rate CO_2 4 mL/min, co-solvent ethanol 96%, the influence of pressure, temperature and amount of added modifier on the phenolic content and antioxidant activity of the extracts were checked. The flow rate of the ethanol used as co-solvent was adjusted to desired concentration of 15% and 20% (*v/v*) to CO_2. The best results were obtained using SC-CO_2 with 20% (*v/v*) ethanol as a modifier, 70 °C and 300 ba (Fig. 2, 3; next page).

HPLC-MS/MS is very useful technique for separation and identification of compounds extracted from complex matrix. We implemented tandem mass spectrometry (MS/MS) for identification of several flavonoids and other phenolic compounds of *M. sativa* leaves extract, such as: apigenin, luteolin, quercetin, biochanin a, kaempferol, caffeic acid and sinapic acid (Fig. 4, page 63). In the Table 2 multiple reaction monitoring transitions (product ion → precursor ion),

Table 2
Multiple reaction monitoring analysis of investigated compounds in the HPLC-QqQ-MS analysis (ion mode negative, collision enegry 35 V).

Compound	Precursor ion *m/z*	Product ion *m/z*
Apigenin	269	117.30
Luteolin	285	133.10
Biochanin a	283	211.15
Quercetin	301	227.05
Kaempferol	285	255.15
Caffeic acid	179	134.25
Sinapic acid	223	121.20

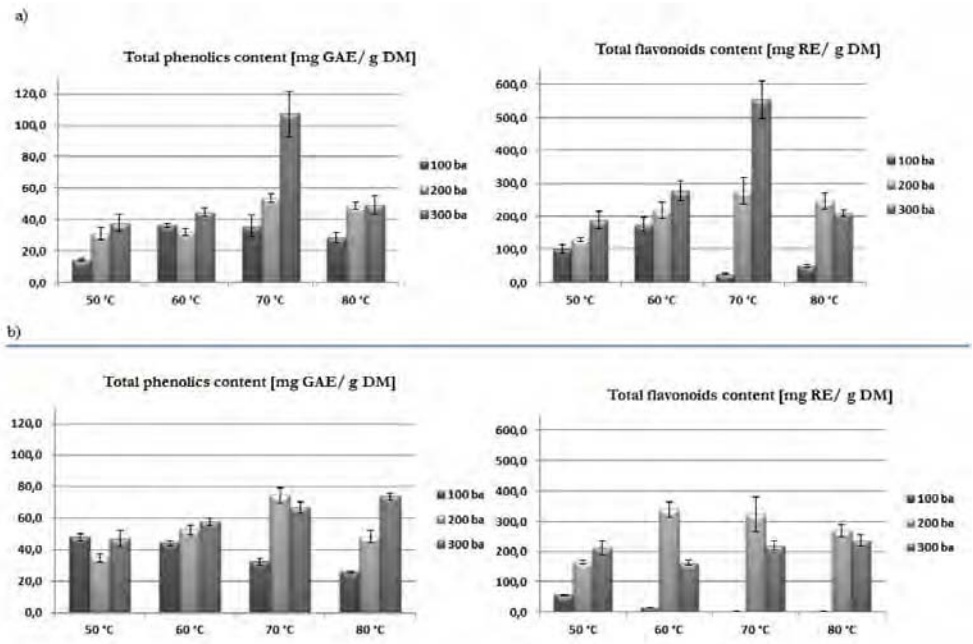

Fig. 2 Total phenolics and flavonoids content in *Medicago sativa* leaves extracts obtained with using different temperature and pressure conditions using SC-CO_2 with (a) 20%, and (b) and 15% (*v/v*) ethanol as a modifier.

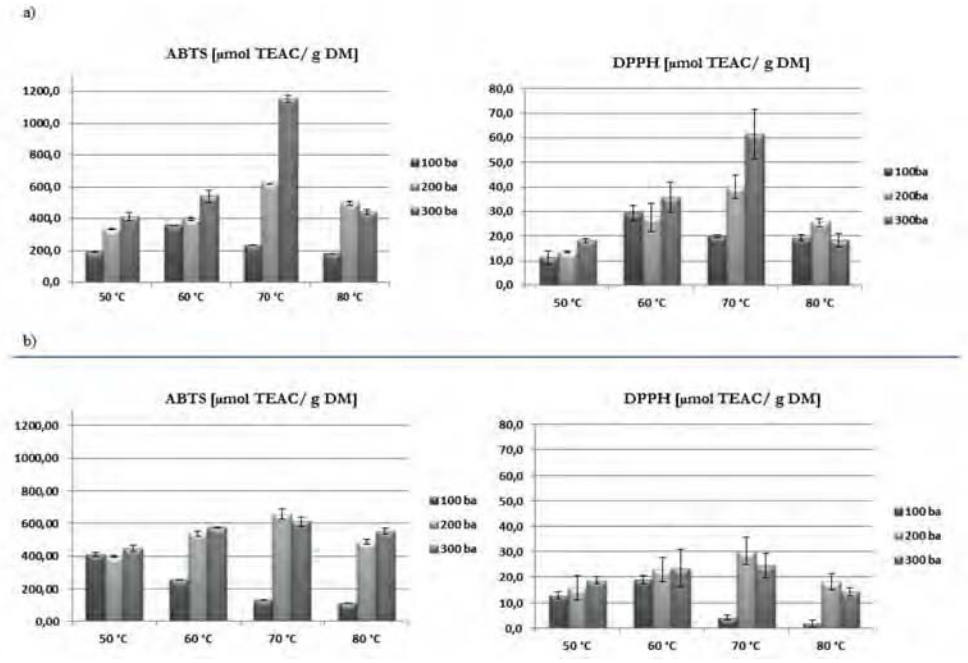

Fig. 3 Antioxidant potential of *Medicago sativa* leaves extracts obtained with using different temperature and pressure conditions using SC-CO_2 with (a) 20%, and (b) and 15% (*v/v*) ethanol as a modifier.

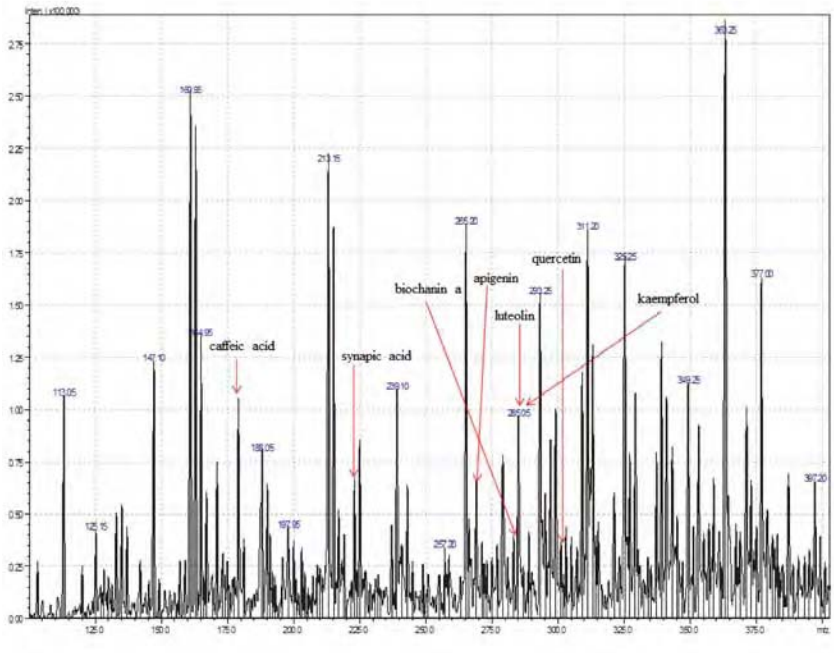

Fig. 4 Mass spectrum of *Medicago sativa* leaves extract from SFE process.

collision energy of these seven identified compounds in *M. sativa*, are summarized. The negative ion mode is more suitable for flavonoids. The MS analysis gave characteristic quasi molecular ions of apigenin ($[M-H]^-$ ion at *m/z* 269), luteolin ($[M-H]^-$ ion at *m/z* 285), quercetin ($[M-H]^-$ ion at *m/z* 301), biochanin a ($[M-H]^-$ ion at *m/z* 283), kaempferol ($[M-H]^-$ ion at *m/z* 285), caffeic acid ($[M-H]^-$ ion at *m/z* 179), sinapic acid ($[M-H]^-$ ion at *m/z* 223).

4. Conclusion

To produce a high quality extract for use in cosmetic and pharmaucetical industry, alternative SFE method for extraction of extract phenolic compounds and flavonoids from *Medicago sativa* was developed. Due to the polarity of phenolic compounds and flavonoids, the addition of a modifier to SC-CO_2 is needed. SFE with 96% ethanol as a modifier allowed to obtain extracts characterized by particularly high concentrations of biologically active compounds. Our studies showed that the best conditions of the extraction of flavonoids, the major contributors to the antioxidant activity of *M. sativa* were 70 °C, 300 ba and using SC-CO_2 with 20% (*v/v*) ethanol as a modifier.

Acknowledgments

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GC-MS and QuEChERS as an Analytical Tool for the Determination of Impurities in Breast Milk

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Keywords
gas chromatography
human milk
polychlorinated biphenyls
QuEchERs technique

Abstract
Human milk is as a very significant factor contributing to proper development of children. Unfortunately milk can also carry polychlorinated biphenyls (PCBs). These compounds are highly harmful and they have negative impact on infants, whose immunological system is still developing. Thus checking milk for these compounds is of great importance. The aim of this study was to develop a suitable method of quantitative and qualitative analysis. We used gas chromatography with mass spectrometry (GC/MS). For sample preparation we used the QuEChERS technique.

1. Introduction

Human milk contains a lot of special nutrients, i.e. proteins, lipids, minerals, and vitamins. Unfortunately milk can also carry harmful substances [1, 2]. Polychlorinated biphenyl (PCBs) are lipophilic compounds, accumulating in fatty tissues, and in this way PCBs can be found in milk. One of the most important sources of exposure is mother's diet; in particular, diet rich in fish products. When pregnant or breastfeeding women want to get appropriate amounts of Ω-3 fatty acids (e.g., DHA), they often eat fish, whose tissues have accumulated environmental contaminants through mostly through feeding on lower organisms. Thus women may be exposed to such organic contaminats as PCBs. Long-term exposure to these substances can lead to serious health problems such as skin diseases, endocrine and reproductive disorders, and neurological problems; particularly in infants, whose immunological system is still developing and does not have defense mechanisms against the potential threat [3, 4].

The method used for determination of PCBs in most cases is gas chromatography with different detection techniques. However, in the recent years the most popular choice is gas chromatography coupled to mass spectrometry [5, 6].

The crucial step in the analytical procedure is sample preparation. Due to low concentration levels of these analytes, it is necessary to use a specific and selective method for isolating, enriching and purifying milk samples. For determining compounds in such matrices as milk, scientists use methods such as classical liquid-liquid extraction (LLE) [6, 7], solid phase extraction (SPE) [8] or accelerated solvent extraction (ASE) [9]. An interesting approach is also the QuECHERs technique [10].

This study aims at determining the PCB (No. 28, 52, 101, 138, 153, 180) content with gas chromatography coupled to mass spectrometry (GC-MS) and developing a sample preparation method used a QuEChERS techniqe.

2. Experimental

2.1 Reagents and chemicals

The following reagents were used in this study: hexane and acetone for GC (POCH, Poland); deionized water (Mili-Q Reagent Water, Merck); magnesium sulphate anhydrous, sodium chloride (POCH, Poland), sodium citrate monobasic, sodium hydrogen citrate sesquihydrate (Sigma-Aldrich); Bondesil PSA, 40 μm (Agilent Technologies) and Bakerbond octadecyl (C18 40 μm, 60 Å). Standards for PCBs No. 28, 52, 101, 118, 138, 153 and 180 came from Dr Ehrenstorfer.

2.2 Instrumentation

The method used was gas chromatography coupled to mass spectrometry (Agilent Technologies, model 6890N). Analytes were separated with Zebron ZB-5MS Guar (30.0 m × 250 mm × 0.25 mm). Flow rate was 1.1 mL/min, using helium as a carrier gas. Column temperature was programmed as follows: 60 °C (1.0 min) → 20 °C/min → 170 °C (0.30 min) →10 °C/min → 310 °C (1.20 min). The injector temperature was 265 °C. The injection volume was 1 μL. The mass ion source temperature was 300 °C.

2.3 Sample preparation method development

First, we selected the method proposed by Luzardo et al. [10] to check the recovery and reproducibility of PCBs from milk. 5 ml of milk (in a 50 ml Falcon tube) was contaminated with standard solution (10 μg/L). Then 5 mL H_2O was added, followed by 10 mL acetonitrile saturated in *n*-hexane. The sample was allowed to rest at room temperature for 30 min. The next step was adding the salts: 4 g $MgSO_4$, 1 g NaCl, 1 g $C_6H_6Na_2O_7.1{\cdot}5H_2O$, and 0.5 g $C_6H_7NaO_7$. The tube content was shaken (1 min) and centrifuged (5 min, 5000 rpm). The extract was transferred to a glass tube. The residue in the Falcon tube was extracted again by adding 5 mL acetonitrile saturated in *n*-hexane. Then the upper layer was added to

Table 1
Limit of detection and quantification and linearity of the method.

PCB	$t_i \pm SD$ / min	*LOD* / μg L^{-1}	*LOQ* / μg L^{-1}	Range of concentration / μg L^{-1}	R^2
28	12.112±0.002	0.22	0.74	2–10	0.997
52	12.733±0.002	0.58	1.93	2–11	0.999
101	14.339±0.002	0.39	1.31	2–10	0.996
118	15.487±0.003	0.40	1.34	2–11	0.998
153	15.884±0.003	0.28	0.93	2–11	0.998
138	16.395±0.002	0.43	1.45	2–11	0.998
180	17.588±0.003	0.49	1.65	2–11	0.998

the first extract. The whole extract was transferred to a Falcon tube with sorbent (500 mg PSA) and salt (900 mg $MgSO_4$). The contents were shaken (1 min) and centrifuged (5 min, 5000 rpm). The extract was evaporated under a stream of nitrogen. The residue was reconstructed with 500 ml *n*-hexane. The sample was then ready for analysis.

The QuEChERS technique was optimized by testing extraction with the use of different solvents (acetonitrile or hexane:acetone 1:1), salts (whether to use citrate salts or not) and sorbents (500 mg PSA or 500 mg PSA + 250 mg C18).

3. Results and discussion

3.1 The method of validation method of the analytical technique

Limit of detection and quantification and linearity of the method are summarized in Table 1. It is evident, that the GC-MS method is linear in the concentration range (2–11 μg/L) with regression coefficients >0.996, and *LOQ* for the analyzed compounds is at a satisfactory level. Typical GC-MS chromatogram of standard solutions of PCBs is depicted in Fig. 1.

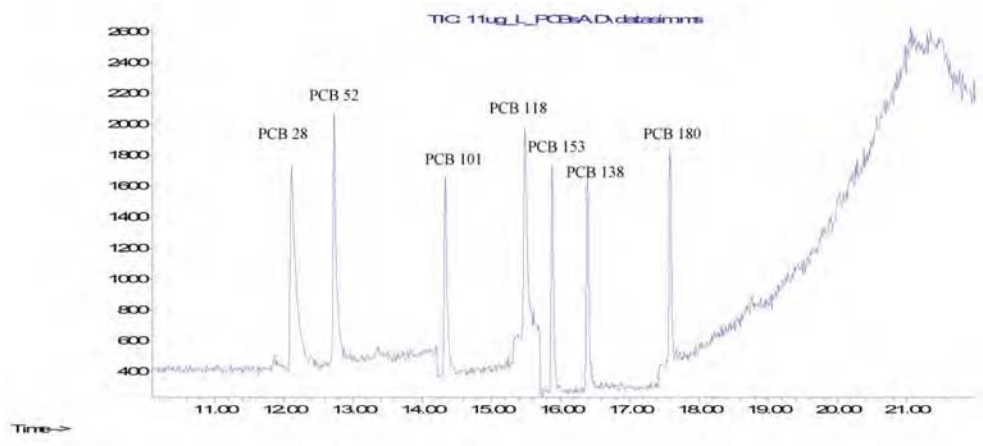

Fig. 1 Chromatogram of standard solutions of PCBs.

Table 2
Efficiency of the extraction process.

PCBs	Recovery / %	SD
28	56.99	11.91
153	60.20	8.96
138	42.56	6.63
180	26.59	6.79

3.2 Sample preparation method

The best results were obtained when we used acetonitrile saturated in *n*-hexane for extraction, the following salts: 4 g $MgSO_4$, 1 g NaCl, 1 g $C_6H_6Na_2O_7 \cdot 1.5H_2O$, and 0.5 g $C_6H_7NaO_7$. and finally the mix of the sorbents containing 500 mg PSA and 250 mg C18. When we used hexane:acetone solvent mixture, a lot of interferents were co-extracted; therefore we chose acetonitrile. The citrate salts and C18 sorbent improve the purity of the extract. The selected extraction method (QuEChERS technique) enabled us to isolate of four PCBs (Table 2).

4. Conclusions

The developed GC-MS method makes it possible to determine test compounds at low concentrations. Isolating analytes from milk is a challenge for the analyst because such matrix is highly heterogeneous (it contains proteins and lipids). The QuEChERS procedure allows us to isolate the four studied compounds. There are many ways to modify this method, so we can improve the efficiency of our version by making changes in the procedure (for example using different sorbents). Dispersive solid phase extraction is the crucial step in this method, because the key is to choose the type and amount of sorbents which will retain the interference, not the analyte.

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New Approach for the Extraction of Pesticide Residues From Cotton Products Using Combined QuEChERS With DLLME

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Keywords
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Abstract
A combination of QuEChERS (quick, easy, cheap, effective, rugged, and safe) and DLLME (dispersive liquid–liquid microextraction) was investigated for pesticide residue analysis in cotton products. Acetonitrile extract, obtained from QuEChERS extraction, was used for DLLME as dispersive solvent. In order to increase the enrichment factor of the extraction procedure, small volume of chloroform was used as extractive solvent. The effect of several extraction parameters, such as type of extractive solvent, volume of extractive solvent, pH, and salt addition was tested. Gas chromatography-mass spectrometry with selected ion monitoring was used for the simultaneous determination of 28 pesticides from various chemical groups exhibiting or suspected to exhibit endocrine-disrupting properties. The proposed method is characterized by the advantages such as small amount of extractive solvent, short extraction time, and good repeatability.

1. Introduction

Cotton is the most important agricultural and industrial crop in the world after food grains and soybean and the most consumed natural fibre in textile and clothing industry. The cotton natural textile fibre is made of the most abundant polymer in nature, cellulose. It consists of (88–95%) pure cellulose, and (3.5–12%) non-cellulose materials, that include proteins, waxes, pectins, inorganics, and other substances [1, 2].

Furthermore, conventionally grown cotton is one of the most by pesticides intensively treated crop. Approximately 60% of all agrochemicals are applied on cotton fields, so cotton has become to the one of the dirtiest agricultural and the most chemical-intensive crops worldwide [2]. During pesticides application, clothing can pick up pesticide residues through spill and drift, then the chemicals can enter the body through the skin. Clothes with higher cotton content pick up more

pesticides. In addition, common laundry procedures do not remove pesticide residues from clothes. There are many accidents that were the evidence of the percutaneous absorption of pesticides from clothes. [2, 3].

The new global concept is to care about textile and clothes safety to improve the protection of the human health and the environment from the harmful pesticide residues. Many governments and organizations are starting to regulate the maximum residue limits (MRL) of pesticides in textile [3].

For textile control, selective and sensitive analytical methods employing sample treatment technique for isolation and pre-concentration of pesticides from the matrix have to be validated. QuEChERS, developed by Anastassiades et al. in 2003 [4], has become one of the most commonly used sample preparation method for the extraction of pesticides from food matrices. The original method involves an initial extraction with acetonitrile and a clean-up step by dispersive solid phase extraction (dSPE). After years, QuEChERS passed by many modifications which include optional steps depending on the sample type. Acetonitrile is used because of the miscibility with water which allowed good penetration into the aqueous fraction of samples, and it can be easily separated from water by adding salts. The major disadvantage of QuEChERS is low enrichment factor obtained [5]. Several attempts had been made to combine QuEChERS with suitable microextraction technique such as DLLME for extraction of pesticides from fruit matrices. DLLME involves ternary component system of extraction employing extractive, dispersive solvents and aqueous phase. In this system, cloudy suspension was formed, which caused a very high contact area between the extraction solvent and the aqueous phase. In the combination of QuEChERS and DLLME, the acetonitrile extract obtained in QuEChERS was used as dispersive solvent [6, 7]. The advantages of DLLME are simplicity of operation, rapidity, less cost, high enrichment factor reached and easy linkage to separation technique such as gas or liquid chromatography. The methods combining QuEChERS with DLLME had been successfully applied for analysis of pesticide residues in complex matrices such as oil-content products [8] or fruits [6].

The current study aimed to investigate the extraction parameters of combined QuEChERS-DLLME method followed by fast GC-MS for pesticide residues in cotton textile samples. The focus on accuracy of the evaluated methodology in dependence on variable extraction parameters was given.

2. Experimental

2.1 Reagents and chemicals

Pesticide standards were obtained from several sources with purity > 95%. The solution of each of 28 pesticides was prepared in acetonitrile at a concentration of 1 mg/mL. The stock solution at the concentration of 20 ng/μL of all pesticides and working standard pesticide mixture solutions with lower concentration were

prepared in acetonitrile. Internal standard triphenylphosphate (TPP) with 99.0% purity was obtained from Fluka. All the solutions were stored at +4 °C. Water for trace analysis and acetonitrile were high-purity-grade solvents for pesticide residue analysis obtained from Merck. Analytical grade anhydrous magnesium sulphate was obtained from Lach-Ner and sodium chloride was taken from laboratory sources. Extractive solvents chloroform, tetrachloroethane, tetrachloromethane were high-purity-grade solvents for GC analysis and were obtained from Sigma-Aldrich. Helium (99.999%) for GC-MS was obtained from Linde.

2.2 Instrumentation

The gas chromatograph 6890 (Agilent) was equipped with an electronically controlled PTV injection port and interfaced to a single quadrupole inert 5975B mass selective detector with an electron ionisation chamber. Capillary column 15 m×0.15 mm I.D., 0.15 μm film thickness CP Sil 8 CB-MS was used for GC separation. The following oven temperature programme was employed: 40 °C (hold 1.75 min), ramped to 150 °C at 60 °C/min, then ramped to 300 °C at 23.8 °C/min and hold for 3.2 min. The total run time was 13.09 min. PTV injector worked in the solvent vent mode employing the temperature program: 20 °C (hold 0.2 min), then ramped at 400 °C/min to 300 °C, hold for 2 min, then ramped at 400 °C/min to 350 °C and maintained for 5 min. Mass spectrometric parameters were set as follows: electron ionisation with 70 eV energy, ion source temperature 250 °C, MS quadrupole temperature 150 °C and MS transfer line temperature 280 °C. The MS system was routinely set in the SIM mode, and for each pesticide, three specific ions were selected and sorted into groups.

2.3 Extraction procedure

Cotton T-shirt was cut out by scissors to the pieces of equal dimensions. 1 g of sample was weighted into a 50 mL centrifuge tube and extracted with 10 mL of acetonitrile. Then, a mixture of 1 g NaCl and 4 g of anhydrous $MgSO_4$ was added and shaken by vortex 1 min. Subsequently, the mixture was centrifuged at 4000 rpm for 5 min. 1 mL of upper layer acetonitrile extract after QuEChERS (dispersive solvent) was transferred into a 25 mL conical centrifuge tube and 120 μL of chloroform (extractive solvent) and 2.5 mL of water were added. The tube was shaken by agitator for 3 min at 1000 rpm and then it was centrifuged. The sediment phase was transferred into the vial and 2 μL of extract were injected to the GC-MS system.

Table 1
List of pesticides and internal standard, their chemical classes, monitored ions, SIM group start times (quantifier ion is in bold).

No.	Pesticide	Chemical class	t_r / min	Ions / *m/z*			SIM group time / min
1	Trifluralin	dinitroaniline	5.454	**306**	264	307	4.00
2	Hexachlorobenzene	OCPs	5.717	**284**	286	282	
3	Carbofuran	carbamate	5.831	**164**	149	131	
4	Atrazine	triazine	5.928	**200**	215	202	
5	Propazine	triazine	5.945	**214**	229	172	
6	Lindan	OPPs	5.968	**181**	183	109	
7	Parathion-methyl	OPPs	6.471	**263**	125	211	6.30
8	Prometryn	triazine	6.597	**241**	184	226	
9	Fenitrothion	OPPs	6.660	**277**	125	260	
10	Malathion	OPPs	6.700	**173**	127	125	
11	Chlorpyriphos	OPPs	6.757	**197**	314	199	
12	Aldrin	OCPs	6.791	**263**	261	265	
13	Parathion-ethyl	OPPs	6.837	**291**	109	139	
14	Dicofol	OCPs	6.911	**139**	111	250	
15	Pendimethalin	dinitroaniline	7.025	**252**	162	281	
16	Bromophos-ethyl	OPPs	7.260	**359**	357	303	7.15
17	*o,p'*- DDE	OCPs	7.311	**246**	248	318	
18	Endosulfan alfa	OCPs	7.408	**241**	239	195	
19	*p,p'*- DDE	OCPs	7.568	**246**	318	316	
20	*p,p'*- DDT	OCPs	7.631	**235**	237	165	
21	Endrin	OCPs	7.791	**263**	265	281	
22	Endosulfan beta	OCPs	7.905	**237**	239	216	
23	*o,p'*- DDT	OCPs	8.191	**235**	237	165	
24	Methoxychlor	OCPs	8.300	**227**	228	274	
25	Bifenthrin	pyrethroid	8.506	**181**	165	166	
26	TPP (internal standard)		8.568	**326**	325	215	
27	Cyhalothrin lambda	pyrethroid	8.848	**181**	197	208	8.7
28	Cypermethrin	pyrethroid	9.723	**163**	165	181	
29	Deltamethrin	pyrethroid	10.380	**181**	253	251	

3. Results and discussion

Several extraction parameters were investigated in this study to achieve optimal recoveries of the extraction of a broader range of pesticides with a suspicion to exhibit endocrine-disrupting properties [9]. The list of studied pesticides, chemical classes, retention times, target and qualifier ions used in SIM mode are shown in Table 1. Pesticides of several chemical groups were searched, namely 11 organochlorine pesticides (OCPs), 7 organophosphorous pesticides (OPPs), 4 pyrethroides, 3 triazines, 2 dinitroanilines, and 1 carbamate.

The initial extraction parameters were set as follows: 1 g of sample for the 1st step of QuEChERS extraction, 1 mL of acetonitrile extract, 100 µL of extractive solvent and 3 mL of water. Type of extractive solvent, volume of extractive solvent, addition of salt and pH were studied.

Table 2
Average area of peaks and the number of pesticides with the optimal extraction recovery depending on the type of extraction solvent.

Extraction solvent	Average area of peaks / A.U.	Number of pesticides with the optimal extraction recovery
Chloroform	91 252	12
Tetrachloroethane	86 667	15
Tetrachloromethane	47 460	18

The choice of extractive solvent is the critical point to achieve efficient DLLME extraction of the pesticides. The proper extractive solvent should have facilities such as higher density than water, extraction capability of the compounds of interest, good chromatographic behaviour and low solubility in water [5]. Chloroform, tetrachloromethane, tetrachloroethane were investigated (with densities 1.48, 1.59, 1.59 g/m^3, respectively). Experiments were performed under the initial extraction parameters. The effect of the solvent type was studied by analysis of three replicates of fortified sample at the concentration level of 250 μg/kg. As it is shown in Table 2, chloroform provides the greatest average peak area of pesticides and 18 pesticides exhibiting recoveries in the interval between 70% and 120%. Furthermore, the use of chloroform as solvent leads to better GC peak shapes of the studied analytes. Therefore, chloroform was selected for the following experiments.

In order to evaluate the effect of extractive solvent volume on the extraction efficiency, the experiments using different volumes of chloroform (50, 60, 80,100, 120, 140, and 160 μL) with a constant volume of the dispersive solvent (1 mL) and water (3 mL) were performed. Increasing volumes of extraction solvent caused an increase in the volume of sedimented phase and decreased enrichment factor. It can be explained by slight solubility of acetonitrile extract in chloroform. 120 μL of chloroform were chosen provided sufficient enrichment factors and satisfactory extraction efficiency.

In the next step, the volume of water (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 mL) was investigated and the volume 2.5 mL of water was chosen.

DLLME is an equilibrium-based extraction approach, and the increase of the ionic strength of the aqueous solution could affect the analyte transfer. Therefore, the effect of salt addition on the analytical response was investigated at the different mass concentration of salt in the range of 0–6% (*w/v*). Addition of salt to the sample did not have a significant effect on the extraction efficiency.

At last, the effect of pH was studied in the rage of 2–10. The pH of the sample can affect the extraction efficiency differently, according to the stability of analytes. Generally, the extraction efficiency was decreasing by using higher pH.

4. Conclusions

The analytical method combining QuEChERS and DLLME followed by fast gas chromatography-mass spectrometry was developed for the simultaneous determination of multiclass group of pesticides with endocrine-disrupting properties in cotton based T-shirt. Pesticides were extracted by acetonitrile through a first stage of QuEChERS method and then refined and preconcentrated by DLLME. The effect of extraction parameters, such as selection of extractive solvent and its volume, volume of water, salt and pH effect influencing DLLME were thoroughly tested. By the application of optimal conditions, satisfactory recoveries and enrichment factors were obtained for the majority of pesticides.

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Characteristic of Morpholinium Ionic Liquids as Gas Chromatography Stationary Phases With McReynolds Constants and Activity Coefficients at Infinite Dilution

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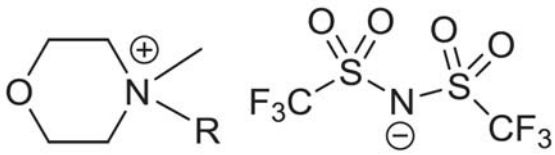
Abstract
Four ionic liquids based on *N*-alkyl-*N*-methylmorpholinium cation ([$Mor_{1,R}$] where *R*= 2, 4, 8, 10) and bis(trifluoromethanesulfonyl)-imide anion [TFSI] were synthesized. Using gas-liquid chromatography a number of parameters describing the sorption properties of the investigated ionic liquids were determined. The values of Kovats indices, McReynolds constants, and activity coefficients at infinite dilution were the basis for the evaluation of intermolecular interactions. The effect of the chain length of the alkyl substituent in the cation, which was used for modification of their polarity has been discussed.

1. Introduction

Ionic liquids (ILs), known as molten organic salts. A typical ionic liquid consists of a large non-symmetrical organic cation and a smaller organic/inorganic anion. ILs are characterized by a unique set of physicochemical properties which include: negligibly low vapor pressure, broad temperature range in the liquid state, thermal stability, high viscosity and density, and affinity to organic or inorganic compounds. Additionally, it is possible to control their physicochemical properties, and thus, to obtain an IL for specific application. This is why they are called "designer solvents" [3]. These properties make their application in many fields of science and technology more attractive [4–6]. Due to the unique properties inherent to ILs, they have been widely applied in various analytical applications including gas chromatography [5]. ILs became very attractive subject in GC development, mostly due to their liquid state, high thermal stability and low vapor pressure. Several studies have been carried out on new ILs and their retention



[$Mor_{1,2}$] [TFSI] R = -CH_2CH_3
[$Mor_{1,4}$] [TFSI] R = -$(CH_2)_3CH_3$
[$Mor_{1,8}$] [TFSI] R = -$(CH_2)_7CH_3$
[$Mor_{1,10}$] [TFSI] R = -$(CH_2)_9CH_3$

Fig. 1 Molecular structures of studied ionic liquids.

mechanism [8–11]. Selectivity is the main object of interest, since it makes their separation capability slightly different from that of conventional and popular stationary phases. The polarity and/or selectivity of IL-based GC stationary phase can be estimated using some empirical measures: e.g., the McReynolds' method [12], the solvatochromic effect of Reinchardt's dye [13], and Nile Red [14]. These polarity methods has not been definitive for ILs because they all seem to fall within the same narrow range values [15–17]. Considering the limitation of the "polarity" methods classification system, the solvation parameter model was developed by Abraham and co-workers by utilizing a large number of test probes that are capable of undergoing multiple interactions with stationary phase [18–20]. Similar qualitative information can be obtained from activity coefficients at infinite dilution. This parameter provide a useful tool for solvent selection in separation process.

The main goal of this work was to evaluate four ionic liquids (Fig. 1), namely: *N*-ethyl-*N*-methylmorpholinium bis(trifluoromethanesulfonyl)imide [$Mor_{1,2}$][TFSI], *N*-butyl-*N*-methylmorpholinium bis(trifluoromethanesulfonyl)-imide [$Mor_{1,4}$][TFSI], *N*-octyl-*N*-methylmorpholinium bis(trifluoromethanesulfonyl)imide [$Mor_{1,8}$][TFSI], and *N*-decyl-*N*-methylmorpholinium bis(trifluoromethanesulfonyl)imide [$Mor_{1,10}$][TFSI] as potential stationary phases for gas chromatography. In order to estimate the polarity of investigated ionic liquids, the values of such parameters as Kovats indices, McReynolds constants were determined. For detailed investigation of the specific intermolecular interactions between the solutes and the ionic liquid-based stationary phase, activity coefficients at infinite dilution were determined in temperature range from 313.15 K up to 363.15 K.

2. Experimental

2.1 Reagents and chemicals

All four of the tested ionic liquids were synthesized by our team according to the procedure described in previous paper [21]. The solvents used, i.e., acetonitrile (purity > 99.9% and mass fraction of water $<2\times10^{-4}$%), dichloromethane (> 99.9%), acetone (> 99.9%), and methanol (> 99.9%) were purchased from POCH (Poland). The Chromosorb W HP-DMCS, 80/100 mesh was purchased from Sigma-Aldrich (Germany). The squalane (>99%) used to prepare the reference column was purchased from Sigma-Aldrich, (Germany). The McReynolds test solutes and *n*-alkanes (C_5–C_{14}) were of analytical grade purity and were purchased from Sigma-Aldrich (Germany). Nitrogen (99.999%), as a carrier gas, was purchased from Linde (Poland).

2.2 Instrumentation

Chromatographic analyzes were performed using an Agilent 7890 A chromatograph equipped with a flame ionization detector (Agilent USA). Data was collected and processed using Agilent software. Both the injector port and the detector had a temperature of 523.15 K. Nitrogen (99.999%) was used as carrier gas. Solute samples with a volume of 1 μL were injected using the microsyringe. To provide infinite dilution conditions of a sample the injector port worked in split mode in the range of 1:50 to 1:100. The column was thermostated at a given temperature for at least 20 minutes. All measurements were made three times to determine the repeatability of the obtained results. Dead retention time was determined using methane. The volume flow of the carrier gas was measured using an automatic soap bubble flow meter. The flow rate was maintained between 0.0050 and 0.0200 dm^3min^{-1} depending on temperature and type of compound.

Tested columns were made of stainless steel tubing (304 grade) purchased from Supelco (USA). The columns had a length of 1 m and an internal diameter of 2.1 mm. The detailed description of the experimental procedure of GC-columns preparation used in this work can be found in the literature [22]. The values of McReynolds constants were determined for the temperature of 393.15 K. The values of activity coefficients at infinite dilution were determined for temperatures in range 313.15–363.15 K.

3. Results and discussion

3.1 McReynolds compounds

The studied ionic liquids are based on the bis(trifluoromethanesulfonyl)imide anion $[TFSI]^-$, well-defined in the literature, which, as a component of ionic liquids, increases their hydrophobic properties. The morpholinium ring

Table 1
McReynolds constants determined at 393.15 K.

Stationary phase	Benzene *X*	1-Butanol *Y*	2-Pentanone *Z*	Nitropropane *U*	Pyridine *S*	ΔI_{av}
$[Mor_{1,2}][TFSI]$	777	923	984	1210	1133	1005
$[Mor_{1,4}][TFSI]$	599	755	807	1087	1005	851
$[Mor_{1,8}][TFSI]$	366	538	601	744	711	592
$[Mor_{1,10}][TFSI]$	311	398	509	657	609	497
SLB-IL111[a]	766	930	957	1192	1093	988
SLB-IL100[b]	602	853	884	1017	1081	887
SLB-IL59[c]	338	505	549	649	583	525

[a] 1.5-Di(2.3-dimethylimidazolium)pentane bis(trifluoromethanesulfonyl)imide.
[b] 1.9-Di(3-vinylimidazolium)nonane bis(trifluoromethanesulfonyl)imide.
[c] 1.12-Di(tripropylphosphonium)dodecane bis(trifluoromethanesulfonyl)imide.

substituted with *n*-alkyl chain $[Mor_{1,R}]^+$ ($R = 2, 4, 8, 10$) was used as the cation. The six-membered ring contains oppositely placed oxygen and nitrogen atoms in its structure. The structural formula of the studied ionic liquids is shown in Fig. 1.

Table 1 presents the determined McReynolds constant values as well as their mean values for each of the four tested columns. Mean values are often used to visualize the overall stationary phase polarity, while the individual component give a valuable evaluation of character of the interactions of a given type. For comparison, McReynolds constant values for three commercially available gas chromatography columns [23] are shown in Table 1. Although these values have been determined for polymerized ionic liquids, they may be a reference point in evaluating the polarity of the tested ionic liquids.

According to the posited classification of stationary phase polarity, the average values of McReynolds constants ΔI_{av} above 400 indicate high polarity. In the described cases, these values are significantly higher. In the case of the ionic liquid with *n*-decyl cation substituent, the mean value of ΔI_{av} was 497, while the highest value determined for the column with the lowest length of the alkyl substituent was twice as high –1005.

In the latter case, the stationary phase polarity is comparable to the value determined for the SLB-IL111 column, whose cation contains two imidazolium rings connected with an *n*-pentane bridge. The elongation of the cation substituent to $n = 10$ results in a decrease in polarity below the value of the least polar commercial phase based on ionic liquid with tripropylphosphonium cation with an *n*-dodecane "bridge" which ΔY_v value is 525. Thus, it can be stated that ionic liquids based on morpholinium cation are tunable in a wide range of their polarity. There is a nonlinear relationship between the McReynolds constants and the number of carbon atoms of the cation substituent – the increase in number of carbon atoms reduces the polarity to an ever lesser degree.

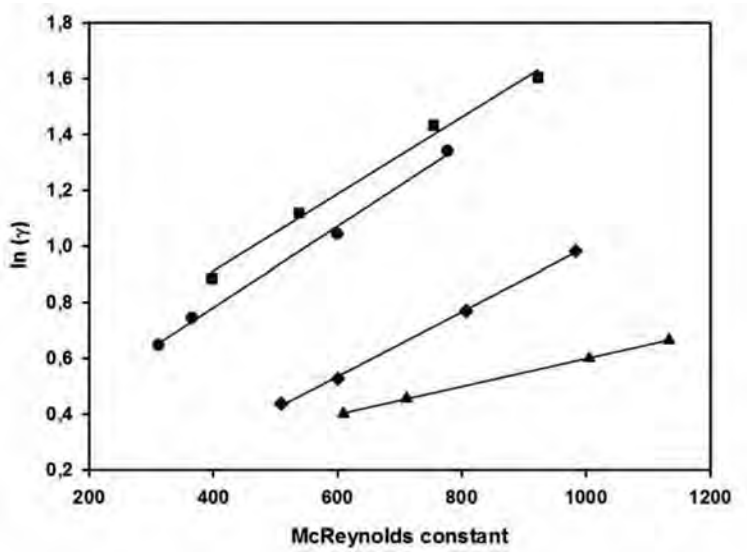


Fig. 2 Plot of dependence of $\ln(\gamma^{\infty}_{1,3})$ as a function of McReynolds constants for the tested compounds at 393.1 K (●) benzene, (■) 1-butanol, (◆) 2-pentanone, (▲) pyridine.

3.2 Activity coefficients at infinite dilution

The Kovats indices discussed in the previous section and the associated McReynolds constants provide only a brief overview of the specifics of intermolecular interactions between the solutes and the stationary phase of the chromatographic column. Their main advantage is the simplicity of both the experimental part and data processing. Nevertheless, for a better description, the values of activity coefficients at infinite dilution $\gamma^{\infty}_{1,3}$ for the four McReynolds compounds and hexane as the *n*-alkane representative were determined (due to the lack of critical values for nitropropane, the compound was omitted). The coefficient values for the tested ionic liquids were determined at seven temperatures: 313.15–363.15, and 393.15K.

The values of activity coefficients at infinite dilution determined for hexane are many times greater than those of McReynolds compounds. The low $\gamma^{\infty}_{1,3}$ values for the McReynolds compounds indicate their greater strength of interaction with the solvent, and thus higher mutual solubility. Comparison of $\gamma^{\infty}_{1,3}$ values of McReynolds compounds shows a slight difference when compared to the previously determined constants: $\gamma^{\infty}_{1,3}$ values decrease in the series 1-butanol > benzene > 2-pentanone > pyridine. Correlation of both parameters is shown in Figure 2.

For the tested compounds, the relationship between the two parameters is approximately linear. In the case of pyridine, which has a high dipole moment and a compact molecular structure, the effect of alkyl chain elongation significantly increases the value of McReynolds constants with relatively little effect on the value of the activity coefficients at infinite dilution. It can be concluded that in the

case of 1-butanol and 2-pentanone, the presence of a long carbon chain results in an increase in solute-ILs interactions, and thus a greater decrease in $\gamma^{\infty}_{1,3}$ value with the elongation of the alkyl substituent of the cation.

4. Conclusions

Based on the results of inverse gas chromatography, a number of parameters were determined describing the sorption properties of new class of ionic liquids based on N-alkyl-N-methylmorpholinium cation and bis(trifluoromethanesulfonyl)-imide anion. The determined values of Kovats indices, McReynolds constants, and activity coefficients at infinite dilution were the basis for the analysis of intermolecular interactions and the effect of the chain length of the alkyl substituent of the cation, which was used as a modulator of their polarity. The ionic liquids tested have a relatively high polarity, as evidenced by the high mean values of McReynolds constants. Correlation of the values of activity coefficients at infinite dilution with temperature inversion provided additional information on how the McReynolds compounds and hexane interact with the tested ionic liquids. Due to the ability of the test compounds to enter into specific intermolecular interactions, the dependence of the solute-ionic liquid interaction strength on the length of the alkyl chain substituent of the cation was determined.

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Development and Characterization of a Miniaturized Injection Cell for the Hyphenation of Electrochemistry-Capillary Electrophoresis-Mass Spectrometry

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Keywords
capillary electrophoresis
disposable electrodes
electrochemistry
mass spectrometry
non-aqueous systems

Abstract
As the mechanistic characterization of the electrochemical behaviour of compounds of interest is important for many applications, sophisticated techniques for the hyphenation of electrochemistry with powerful separation and detection systems are in demand. In this context, important aspects are flexibility, consumption of solvents as well as sample solutions, and a fast detection of electrogenerated species. For this purpose, a miniaturized injection cell for the online coupling of electrochemistry, capillary electrophoresis and mass spectrometry (EC-CE-MS) was developed. Due to the small cell volume, low amounts of sample solution in the range of few microliters are sufficient. The integration of disposable thin-film electrodes leads to high flexibility concerning electrode materials. Because of the materials used in the cell design, the system can be used for the investigation of aqueous and non-aqueous solutions. Thus, the concept of online EC-CE-MS is extended to a broad range of analytical problems.

1. Introduction

Electrochemistry is a fundamental technique in chemical research. It is frequently applied in material sciences such as corrosion studies [1], the development of energy carriers [2], or in the development of microbial fuel cells [3]. But electrochemistry is also important for bioanalytical studies, for example the simulation of oxidative stress [4, 5], or metabolic processes [6].

Since introduced by Esaka et al. [7], the hyphenation of electrochemistry and CE is a sophisticated method for the analysis of the electrochemical behavior of charged species. Additionally to data of voltammetric measurements, the migration behavior of electrogenerated species can be helpful in the interpretation of structural features and state of charge of oxidation or reduction

products. Electrochemistry as a sample preparation technique for CE was introduced by Matysik in 2003 [8]. At the so-called electrochemically assisted injection, analyte molecules were electrochemically modified in a special injection cell and then analyzed by CE with UV/VIS detection. Hyphenation of EC-CE-MS was demonstrated by Scholz and Matysik [9] using different ferrocene derivatives. Neutral compounds could be separated without additives by conversion into charged species. In contrast to the setups above, which are based on classical electrochemical cells with 3-electrode configurations, Palatzky et al. [10] developed a fully automated device for online EC-CE-MS based on disposable screen-printed electrodes (SPEs). This offers several advantages like lower sample consumption compared to classical cells, easy exchange of electrodes avoiding time-consuming cleaning procedures, and high flexibility concerning electrode materials. The EC-CE-MS device of Palatzky et al. being designed for SPEs, it cannot be applied to non-aqueous solutions, as SPEs are not solvent-resistant. This issue can be overcome by using thin-film electrodes. However, in this case it is not possible to apply sample droplets directly onto the electrodes. Spreading of droplets due to the low surface tension of organic solvents leads to shortcuts, corrosion of electrical contacts and dissolution of plastic parts of the setup. Thus, a miniaturized injection cell for thin-film electrodes was developed and characterized with ferrocene derivatives to enable EC-CE-MS experiments under non-aqueous conditions.

2. Experimental

2.1 Reagents and chemicals

The following chemicals were used, all of analytical grade or higher: acetic acid (Sigma Aldrich, USA), acetonitrile, ammonium acetate, decamethylferrocene (ABCR, Germany), formic acid (Merck, Germany), ferrocene (Riedel-de-Haën, Germany), ferrocenemethanol (ABCR, Germany), isopropanol (Roth, Germany).

2.2 Instrumentation

For EC-CE-MS measurements the laboratory-constructed CE system developed by Palatzky et al. [10] was used. The setup was installed in a plexiglass box and connected to a laboratory-constructed high voltage supply. A micrOTOF time-of-flight mass spectrometer (Bruker Daltronics, Germany), equipped with a coaxial sheath liquid ESI interface (Agilent Technologies, Germany) was used for detection. It was operated in positive ion mode. A mixture of isopropanol:water:formic acid (49.9:49.9:0.2, *v/v/v*) was added as sheath liquid at a flow rate of 8 µL/min with a syringe pump. Separations were carried out in fused silica capillaries (Polymicro Technologies, USA) with an outer diameter of 360 µm, an inner diameter of 25 µm, and a length of 35 cm. The detection end of the

capillary was polished to a plane edge while the injection end of the capillary was polished to an angle of 15°. The capillaries were preconditioned by flushing with 0.1 M NaOH for 10 min, followed by water for 5 min and ACN/10 mM NH_4OAc/1 M HOAc for at least 30 min. A thin-film electrode with gold working, counter, and quasireference electrode (ED-SE1-Au, Micrux Technologies, Spain) was used for the oxidation. The electrode was installed in the new injection cell described in detail in section 3.1.

As model system, a solution of 1.5 mM ferrocene (Fc), 1 mM ferrocenemethanol (FcMeOH), and 40 µM decamethylferrocene (dMFc) in ACN/10 mM NH_4OAc/1 M HOAc was used. For the CE protocol, 8 µL of sample solution were filled into the cell. For hydrodynamic injection into the CE system, the tapered end of the separation capillary was automatically placed onto the working electrode surface for 2 s. After the injection, the capillary was placed into the 2 mL buffer reservoir and the separation voltage of 18 to 26 kV was applied. Separations were carried out in ACN/10 mM NH_4OAc/1 M HOAc without previous oxidation and after oxidation at 0.5 V vs. the gold quasireference electrode for 30, 60, and 90 s. For data evaluation the corresponding mass signals *m/z* 186.01 (Fc), *m/z* 216.02 (FcMeOH), and *m/z* 326.20 (dMFc) were extracted.

The parameters for the MS detection were as follows. Acquisition: ion polarity: positive; mass range: 100–350 *m/z*; spectra rate 5 Hz. Source: end plate offset: –500 v; capillary: –4000 V; nebulizer: 1.0 Bar; dry gas: 4.0 L/min; dry temperature: 190 °C. Transfer: capillary exit: 75.0 V; skimmer 1: 25.3 V; hexapole 1: 23.0 V; hexapole RF: 65.0 Vpp; skimmer 2: 23.0 V; lens 1 transfer: 38.0 µs; lens 1 pre puls storage: 6.0 µs.

3. Results and discussion

3.1 Design of the injection cell

The design of the injection cell had to fulfill different demands. To enable measurements in non-aqueous media, all materials had to be resistant against organic solvents. Additionally, the cell geometry had to be adapted to the EC-CE-MS setup. A schematic illustration of the final injection cell is shown in Fig. 1.

The cell body was made of polyetheretherketone (PEEK), a highly chemical resistant and mechanically stable material. To prevent leakage, a sealing ring was integrated at the bottom of the cell chamber. As commercial materials such as NBR or Viton are attacked by organic solvents, a sealing ring was prepared out of a silicone tube with appropriate dimensions. The electrical contact to the integrated Micrux thin-film electrode was achieved by spring contact probes. To allow a fast assembling and disassembling of the cell, magnets were integrated to close the cell. To summarize, a miniaturized electrochemical cell with integrated thin-film electrodes was designed, which is suitable for electrochemical experiments in aqueous and non-aqueous solutions and hyphenation to online CE.

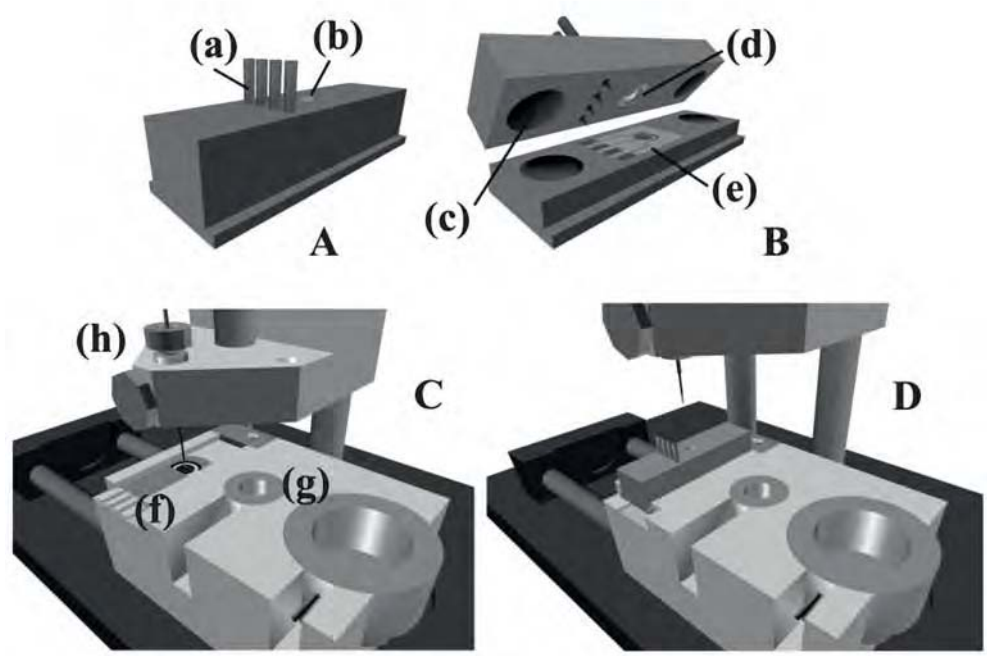

Fig. 1 Illustration of the injection cell: (A) closed cell, (B) open cell, (C) EC-CE setup with SPE, (D) EC-CE setup with injection cell. The cover and the bottom of the cell are manufactured of PEEK. Electrical contact of the electrode is achieved by spring contact probes (a). The tubular shaped electrochemical cell (b) is open at the top to enable an injection with the separation capillary of the CE system. The cell is closed by magnets (c). A silicone sealing ring (d), made of a silicone tube with an inner diameter of 2 mm and a wall thickness of 1 mm is delimiting the cell and prevents leakage. Commercial thin-film electrodes (e) (Micrux Technologies, working electrode diameter 1 mm) are inte-grated into the cell. The injection cell can be installed in the CE setup instead of a SPE (f). The injection capillary (h) can be moved between electrode and buffer reservoirs (g).

3.2 EC-CE-MS experiments

For EC-CE-MS experiments with the developed cell, small sample volumes of 10 µL or lower were sufficient. By using separation capillaries with small inner diameters and high separation voltages, a fast separation and detection of cationic and neutral species is possible as Fig. 2 (next page) shows. Separations were carried out at different separation voltages without previous oxidation using a model mixture of Fc/FcMeOH/dMFc.

A detection of cationic and neutral species was possible within 180 s when a high voltage of 26 kV was applied, so that a fast detection of oxidation products, especially cationic ones, can be achieved. dMFc was only detected as cationic species, as it is easily oxidized by oxygen. FcMeOH was only detected as neutral species, whereas Fc can barely be detected. After previous oxidation, the cationic species of ferrocene and ferrocenemethanol were formed, as shown in Fig. 3 (page 87).

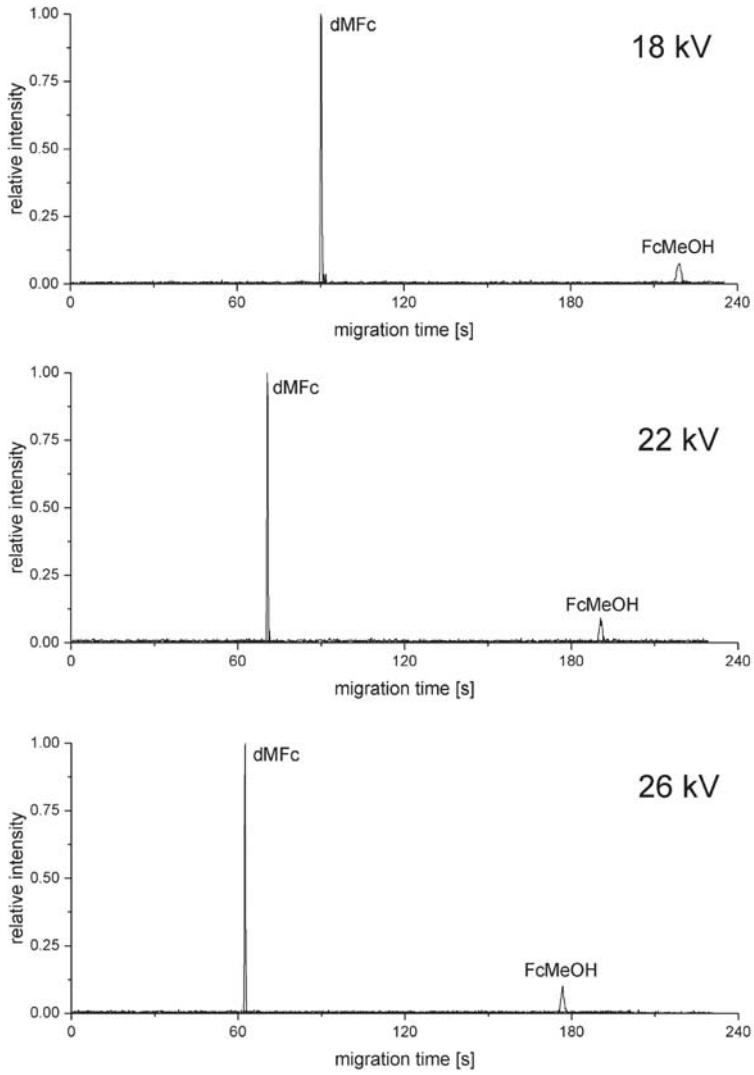

Fig. 2 Electropherograms of the model mixture Fc/FcMeOH/dMFc. dMFc easily gets oxidized by traces of oxygen in the solvent, so only the cationic species is detected with high intensity. FcMeOH is detected as neutral species with a low intensity and is migrating with the EOF. Fc is too hydrophobic to be ionized in ESI, so that it is not detectable without previous oxidation. A fast detection of cationic and neutral species within 180 s is possible, when a separation voltage of 26 kV is applied.

Fc itself being not detectable in ESI-MS, the detection of Fc^+ was possible with increasing oxidation time. At the same time, the cationic $FcMeOH^+$ was formed. Overall, short oxidation times were sufficient for the detection of the oxidation products.

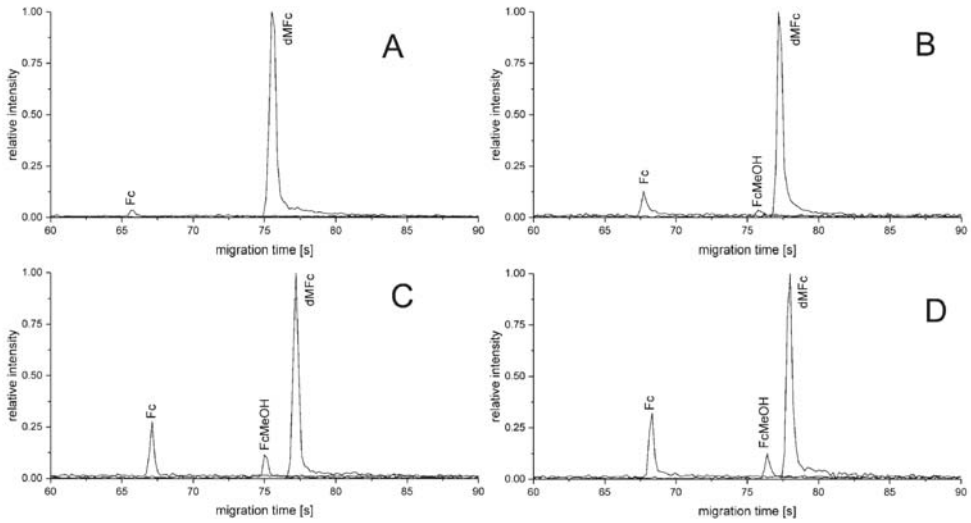

Fig. 3 Electropherograms of the model mixture Fc/FcMeOH/dMFc without (A) previous oxidation, and after oxidation for (B) 30, (C) 60, and (D) 90 s at 0.5 V on a Micrux thin-film gold electrode. Without oxidation, $dMFc^+$ is the main species. Only traces of the Fc^+ are detected. With increasing oxidation time, the amount of Fc^+ and $FcMeOH^+$ increases. Injection was carried out by placing the injection capillary onto the electrode surface for 2 s. Separations were carried out at 18 kV.

4. Conclusion

In conclusion, a miniaturized injection cell for EC-CE-MS was designed capable of handling very small amounts of sample solution. In online CE-MS analysis, a fast detection of the cationic ferrocene and ferrocenemethanol species was possible after short oxidation times. Next to the miniaturization, another advantage of the cell design in combination with solvent-resistant thin-film electrodes is the compatibility to non-aqueous solutions. Thus, online EC-CE-MS measurements are possible without the problems of the solutions spreading into electrical contacts or dissolution of electrode layers, as it would be the case by application of screen-printed electrodes. This extends the applicability of online EC-CE-MS to analytes that are only soluble in organic solvents. The integration of the disposable sensors leads to a high flexibility in electrode materials and to an easy exchange of electrodes, which is minimizing artifacts due to adsorption or electrode fouling. This makes the setup attractive for further applications.

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Forced Convection in Scanning Electrochemical Microscopy Introduced by an Electrochemical Flow Cell

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Keywords
electroanalytical chemistry
electrochemical flow cell design
forced convection
instrumental analysis
scanning electrochemical microscopy (SECM)

Abstract
In the frame of the contribution, the effects of forced convection on scanning electrochemical microscopy (SECM) experiments are presented. The convection is generated by a flow of the mediator solution through an electrochemical flow cell, developed especially for this purpose. A description of the mandatory design aspects of the experimental flow cell setup is also included. Using a macroscopic working electrode, the flow of the SECM electrolyte leads to the formation of a stable diffusion layer during a chronoamperometric measurement in contrast to a growing diffusion layer in quiescent solution. To characterize the effects of the forced convection, the diffusion layer around the inserted platinum substrate electrode was investigated utilizing chronoamperometric measurements and SECM imaging. In summary, a prototype of an electrochemical flow cell for SECM was developed, which enables steady state diffusion layer characteristics at a macroscopic working electrode and thus offers interesting possibilities for future applications.

1. Introduction

Scanning electrochemical microscopy is a versatile analytical method for imaging the (electro)chemical activity and topographic details of a surface [1]. The non-invasive method facilitates high spatial resolution and can be used to investigate both conducting and non-conducting material [2]. It is a part of scanning probe microscopy techniques and is therefore closely related to atomic force microscopy or electrochemical scanning tunneling microscopy, for example [3].

In SECM a miniaturized electrode, which is termed ultramicroelectrode (UME), is utilized as a probe. Information can be obtained via reactions at the UME, which is scanned across the surface area of interest in solution. Fig. 1 (on the next page) illustrates the setup of the instrument schematically.

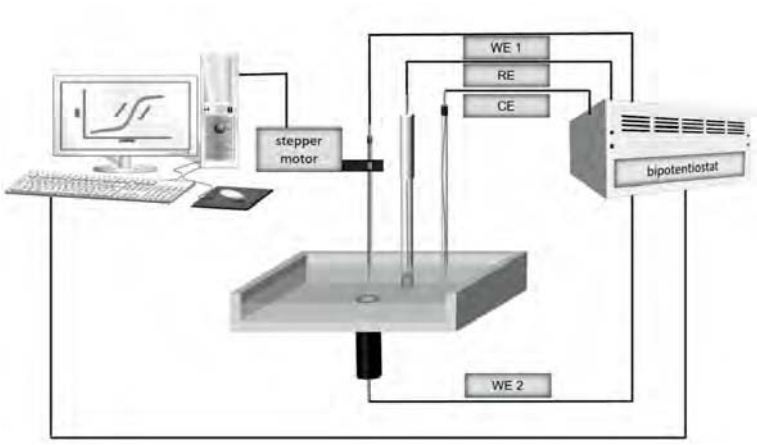

Fig. 1 Schematic setup of the SECM. The probe, which acts as working electrode 1 (WE 1), is scanned across a surface (WE 2) via stepper motors. The electrochemical cell is completed by a reference electrode (RE) and a counter electrode (CE). The measurements are performed with a bipotentiostat controlled by a computer.

When operating the SECM in the amperometric substrate generation-tip collection (SG/TC) mode, the species to be recorded at the UME (collector electrode) is initially generated at a second working electrode (generator electrode) placed below [2, 4]. Problems arise if a macroscopic generator electrode is used due to the time-dependent growing diffusion layer of the generated species. This leads to a continuous change of the conditions at the UME, which is placed within the diffusion layer of the species in a fixed distance from the second electrode.

One new approach concerning the performance of SECM experiments is to introduce forced convection to the system by stirring the electrolyte solution, as previously established by our research group [5]. Forced convection leads to an enhanced mass transfer, which results in the formation of a stable time-independent diffusion layer with a defined thickness around a macroscopic electrode [6]. Fig. 2 compares the conditions at the UME for SECM experiments in SG/TC mode without and with forced convection.

Thus, by applying forced convection, steady state diffusion layer characteristics at a macroscopic working electrode can be generated, which offers interesting possibilities for future applications. In the frame of the contribution, the convection is generated by a flow of the mediator solution through an electrochemical flow cell, developed especially for this purpose.

2. Experimental

2.1 Reagents and chemicals

For all experiments ferrocenemethanol (c = 1.5 mM; FcMeOH, 99%, ABCR, Germany) was used as electrochemical mediator. An aqueous solution was prepared

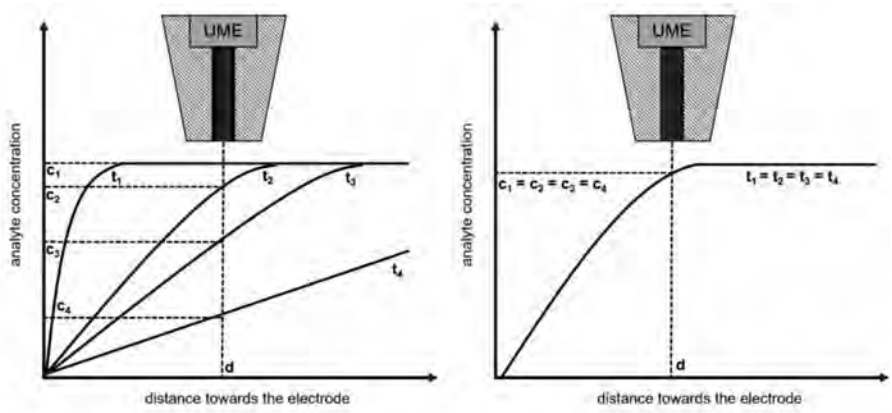

Fig. 2 Comparison of the conditions at the UME during SECM experiments in SG/TC mode without and with forced convection applied to the system.

Fig. 3 Experimental setup. Developed electrochemical flow cell integrated into the SECM instrument.

using Milli-Q water (provided by Milli-Q Advantage A10 system, Merck Millipore, Germany). Potassium nitrate (c = 0.2 M, analytical grade, Merck, Germany) was added as supporting electrolyte.

2.2 Instrumentation

Fig. 3 gives an overview of the most important aspects of the experimental setup with the electrochemical flow cell integrated into the SECM instrument. Starting from reservoir 1, the mediator solution is pumped into the electrochemical cell with an HPLC pump (model HPLC 6400 884, Knauer, Germany), which is placed outside the instrument. A constant flow is achieved as soon as the liquid has contact to reservoir 2 filled with the same solution. The liquid is pumped back from reservoir 2 into reservoir 1 using a peristaltic pump (model IPS 16, Ismatec, Switzerland).

The prototype of an electrochemical flow cell was built by the fine mechanical workshop of the Faculty of Chemistry and Pharmacy at the University of Regensburg. The platinum substrate electrode (d = 2 mm, CH Instruments, Texas) integrated into the cell was polished with 0.3 µm alumina on polishing cloth (TexMed, Lake Bluff) prior to each measurement. A platinum wire was used as counter electrode and an Ag/AgCl/sat. KCl electrode as reference electrode. All potentials mentioned refer to this reference system.

SECM experiments were performed with a commercially available SECM 920C from CH Instruments (Texas). An UME with a diameter of 25 µm fabricated according to Bard and co-workers [7] was utilized for all measurements.

The chronoamperometric response of the macroscopic platinum substrate electrode was recorded for the oxidation of FcMeOH in order to investigate the steady state diffusion layer characteristics at the macroscopic substrate electrode generated by a flow of the mediator solution through the developed cell prototype. The potential of the substrate electrode ($E_{substrate}$) was set to 0.45 V. The sample time was set to 0.1 s, the quiet time was 0 s and the duration of the measurement was 60 s.

For further characterization of the effects of the forced convection, the stability and uniformity of the diffusion layer was studied via hydrodynamic SECM imaging in SG/TC mode. For this purpose, the UME was positioned within the diffusion layer of the substrate electrode (distance to electrode: 29 µm) and an area of 180 µm in x-direction and 40 µm in y-direction was imaged repetitively without and with forced convection, respectively. The probe velocity was set to 100 µm/s with a data point recorded every 6 µm. $E_{substrate}$ was set to 0.45 V for the oxidization of FcMeOH, while a potential of 0 V was chosen for the reduction of the generated species at the UME (E_{probe}). To generate forced convection, the rate of the flowing mediator solution was set to 2.1 ml/min in all experiments as this was the optimum flow rate to ensure a constant liquid level.

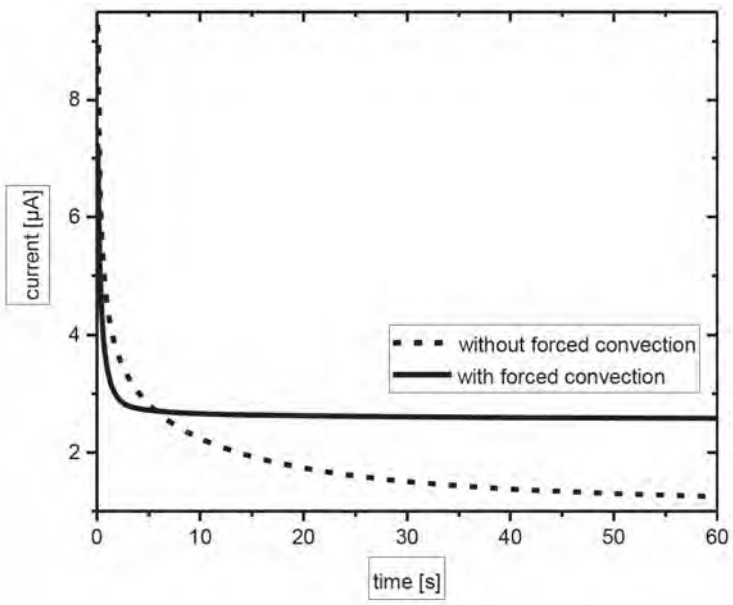

Fig. 4 Chronoamperometric response of the substrate electrode for the oxidation of ferrocenemethanol; $E_{substrate}$ = 0.45 V; quiet time: 0 s; sample time: 0.1 s; flow rate for convection: 2.1 ml/min.

3. Results and discussion

Different cell design concepts and geometries were studied in order to optimize the flow through the electrochemical flow cell and to obtain a laminar flow with a constant liquid level. This was rather challenging in comparison to classical flow cell configurations, as a flow cell for SECM has to be open on top to enable positioning of the UME. The final cell prototype consisting of Teflon is presented in Fig. 3. A constant liquid level is facilitated by the slide at the end of the cell and its continuous contact to the solution in reservoir 2.

To characterize the effects of the forced convection, the diffusion layer around the inserted platinum substrate electrode was investigated utilizing chronoamperometric measurements and SECM imaging.

As can be seen in Fig. 4, the current at the macroscopic electrode for the oxidation of FcMeOH decreases in quiescent solution (dotted line) according to the Cottrell equation [8]. This corresponds to the growing diffusion layer of the generated species. Hence, the concentration gradient of the electroactive species flattens with time resulting in a decreased mass transfer towards the electrode. In contrast, the application of forced convection leads to a constant and increased current corresponding to the formation of a stable time-independent diffusion around the electrode. Thus, steady state diffusion layer characteristics at a macroscopic working electrode can be generated by a flow of the mediator solution.

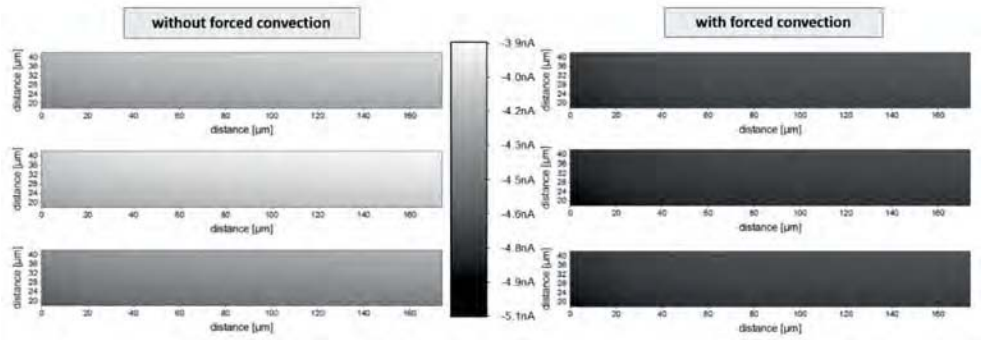

Fig. 5 Three repetitive SECM images within the diffusion layer of the substrate electrode without and with convection in SG/TC mode. $E_{substrate}$ = 0.45 V; E_{probe} = 0 V; scan rate: 100 µm/s; increment: 6 µm; quiet time: 1 s; probe diameter: 25 µm; flow rate for convection: 2.1 ml/min.

To study the stability and uniformity of the diffusion layer, SECM images were recorded repetitively in SG/TC mode both in quiescent solution and with forced convection.

As depicted in Fig. 5 (on the next page) , the images without forced convection differ from each other, corresponding to the growing diffusion layer and the changing conditions at the UME. For measurements with forced convection the current increases due to the increased mass transfer within the system. Furthermore, three almost identical images with a constant current throughout the recorded area are obtained corresponding to a stable diffusion layer. This proves the stability and uniformity of the steady state diffusion layer generated by a flow of the mediator solution.

4. Conclusion

In summary, a prototype of an electrochemical flow cell for SECM was developed and characterized regarding the effects of a flowing mediator solution. It was shown that the flow cell facilitates steady state diffusion layer characteristics at a macroscopic working electrode and thus offers interesting possibilities for future applications such as time-independent measurements in the context of the SG/TC mode or fast scan imaging.

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Optimization of the Extraction Process of Polysaccharides From Yeast *Saccharomyces cerevisiae* Using Response Surface Methodology

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Keywords
extraction
optimization
response surface methodology
yeast

Abstract
Two independent variables of extraction on three levels were optimized using Response Surface Methodology (central composite design, CCD). The goal was to obtain maximum yield of water-soluble polysaccharides using Hot Water Extraction. Polysaccharides were extracted from yeast *Saccharomyces cerevisiae*. The parameters considered for the optimization were temperature (60, 80, or 100 °C) and extraction time (60, 90, or 120 min). The optimal theoretical extraction conditions were found to be: temperature 100 °C and time 60 min. Under these conditions the predicted optimal yield was 376.1 mg and yield from conducted experiment was 378.8 mg.

1. Introduction

Over the past decade, intensive studies on the potential of β-(1,3)-D-glucan have been conducted. β-glucans have very interesting physic-chemical properties, especially gelling ability, which is why they are intensively used in the food industry. In addition, these substances exhibit biological activity (e.g., stimulation of immune system), which is also used in the pharmaceutical, medical and cosmetic industries. They are divided into several classes, and their differentiating factor is the structural characteristics closely related to the biological material in which they are found and from which they have been isolated.

β-(1,3)-D-glucans have been found in both non-nuclear organisms and in higher organisms containing cell nuclei, and can be obtained from cereals, fungi, algae and yeasts. They are the cell wall components of these organisms or plants. The structure of glucan derived from fungi and yeast includes the main straight

chain, composed of β-D-glucopyranose units, linked by (1,3) randomly arranged branches composed of β-D-glucopyranose units bonded by bonds (1,6).

The aim of this study was to obtain maximum yield of water-soluble polysaccharides using Hot Water Extraction. Response surface methodology was used for this task.

2. Experimental

2.1 Reagents and chemicals

Bakery yeast of *Saccharomyces cerevisiae* were bought in local market. All the chemicals used: ethanol, *n*-butanol, and chloroform were of analytical grade.

2.2 Optimization of Hot Water Extraction parameters by RSM

An optimization study for the two parameters (temperature and time) was performed using Minitab 17 (LEADTOOLS). Three-level-two-factor Central Composition Design was employed to optimize the parameters. The independent variables (temperature and time) and their levels followed have been show in Table 1.

The whole CCD design consists of 13 factorial points. The design involves eight randomized points of the independent variables with their responses of dependent variable and three replicates at the centre point to evaluate the pure error. The response can be described by the shown mathematical model

$$Y = \beta_0 + \Sigma\beta_j X_j + \Sigma\beta_{jj} X_j^2 + \Sigma\Sigma\beta_{ij} X_i X_j \quad (1)$$

where Y is the response variable measured for each combination of factorial level; β_0, β_j, β_{jj}, and β_{ij} are the regression coefficients for intercept, linearity, square, and interaction respectively; X_i and X_j are the codes of the independent variables.

2.3 Isolation of polysaccharides

In this study a modified method described in [3] was used. The cubes of yeast *Saccharomyces cerevisiae* were first dried at 60 °C in oven. The yeast was next crushed using a ceramic mortar and pre-treatment with aqueous ethanol (80%) for 24 hours at room temperature. Addition of ethanol excludes some of the constituents: reducing sugar, amino acids, fatty acids and endogenous enzymes. The Hot Water Extraction was carried out for different time periods (60, 90 or 120 min) and temperature (60, 80 or 100 °C) using a water bath (Conbest M2). 10 g of pre-treatment yeast was mixed with 150 ml distilled water. Once the extraction was completed, the extract was left to cool to room temperature. After cooling the mixture was centrifuged (10000 rpm 15 min at 20 °C). The solution

Table 1
Experimental matrix and values of the observed responses.

Runs	Time / min X_1	Temperature / °C X_2	Yield / mg	
			Actual	Predicted
1	60(–1)	60(–1)	198.6	218.8
2	60(–1)	80(0)	329.7	317.4
3	60(–1)	100(1)	384.0	376.1
4	90(0)	60(–1)	272.3	246.0
5	90(0)	80(0)	269.6	297.9
6	90(0)	80(0)	280.1	297.9
7	90(0)	80(0)	294.6	297.9
8	90(0)	80(0)	316.3	297.9
9	90(0)	80(0)	332.1	297.9
10	90(0)	100(1)	280.0	309.7
11	120(1)	60(–1)	363.7	369.9
12	120(1)	80(0)	359.2	374.9
13	120(1)	100(1)	361.7	339.8

was concentrated using a nitrogen. The concentrated sample (25 ml) was subjected to Sevag method (4:1 of $CHCl_3$/*n*-BuOH) to exclude the protein components. After the denaturation, samples were centrifuged (9000 rpm 15 min at 20 °C). The supernatant was mixed with aqueous ethanol 80% (1:1, *v/v*) and left for 24 hours at 4 °C to precipitate polysaccharides. Next to polysaccharides were lyophilized and weighed.

3. Results and discussion

During this study the target of the extraction process was maximize the amount of polysaccharides. In order to explore the relationship between temperature, time (inputs) and extraction yield (output) a RSM method has been applied. Obtained experimental and theoretical results are shown in Table 1.

The results of multiple regression analysis for the values of response obtained from the experiments indicated that the response variable of polysaccharides could be predicted by a quadratic polynomial model within the levels of investigation as represented in equation

$$Y = -363 - 2.46\,X_1 + 16.62\,X_2 + 0.0537\,X_1X_1 - 0.05\,X_1X_2 - 0.0781\,X_1X_2 \qquad (2)$$

where Y is the mass of polysaccharides, X_1 time [min], and X_2 temperature [°C].

It is highly important to test the fit of mathematical model to represent the relationship between the dependent and independent variables. The model fit is summarized in Table 2. *F*-test and analysis of variance (ANOVA) for the model showed that the *p*-value of the model is 0.014. This suggests that model is significant and adequate to predicting the masses of polysaccharides from extraction

Table 2
ANOVA for response surface of the quadratic polynomial model.

Source	Sum of squares	Degree of freedom	Mean of square	*F*-value	*p*-value
Model	26264	5	5252.9	6.58	0.014
Lack-of-fit	2954	3	984.7	1.49	0.344
Pure error	2635	4	658.7		
R^2	0.8245				
R^2_{adj}	0.6992				

Table 3
Estimated regression model.

Source	Sum of squares	Degree of freedom	Mean of square	*F*-value	*p*-value
linear	11034	2	5517.2	6.91	0.022
time (X_1)	4948	1	4947.9	6.20	0.042
temperature (X_2)	6087	1	6086.5	7.62	0.028
Square	6450	2	3225.1	4.04	0.068
X_1X_1	6442	1	6441.8	8.07	0.025
X_2X_2	1105	1	1105.3	1.38	0.278
2-way interaction	8780	1	8779.7	11.00	0.013
X_1X_2	8780	1	8779.7	11.00	0.013

processes. The value of R^2 was 82.45. Literature suggested that good fit should be at least 80% and in our case it was fulfilled. The *F*-value and *p*-value of the lack-of--fit test were 1.49 and 0.344. For a good fitted model a *p*-value of the lack-of-fit should be above 0.05. Therefore the lack-of-fit is non-significant.

Multiple regression analysis results are shown in Table 3. The table involves the significant and non-significant coefficients of model. It could be seen that only square coefficient of X_2^2 is non-significant, rest of terms have a significantly effect on the model.

Fig. 1a shows the 3D surface plot of the interactive effects of the independent variables corresponding to the extraction. Generated surface response has a saddle profile and presents a saddle point as the critical point. The saddle point is an inflexion point between a relative maximum and a relative minimum. To obtain a maximum response to a studied system, the saddle point coordinates do not serve as optimal values but it is possible to find the optimum region through visual inspection of the surfaces. For this task we can use a contour plot (Fig. 1b). In our case the optimal conditions are 60 min 100 °C and 120 min 60 or 80 °C. Taking into account the energy needed to perform the analysis, we chose the first option, 60 min and 100 °C. Under the "optimal" conditions the predicted masses of polysaccharides was 376.1 mg and experimental masses were 380.1, 372.6, and 383.7 mg. Theoretical and real yield are in very good agreement. These outcomes indicate the adequacy of mathematical model to represent the Hot Water Extraction variables.

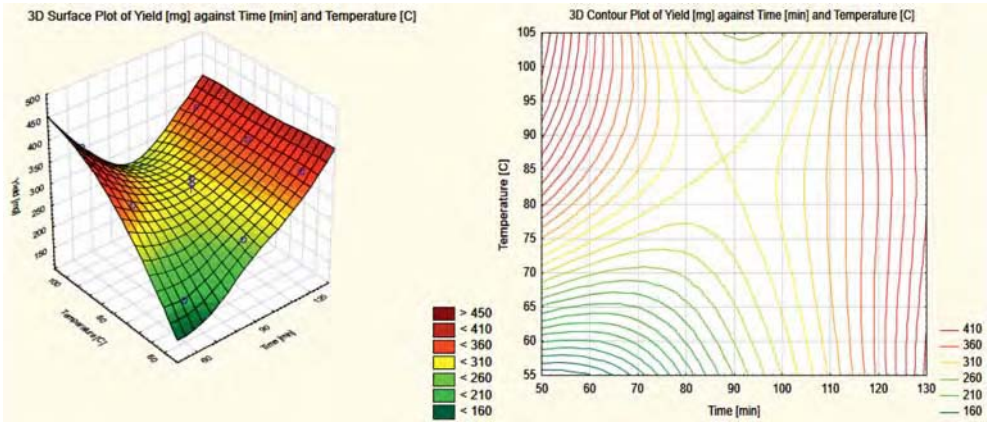

Fig. 1 3D surface plot (left) and contour plot (right) of the interactive effects of the independent variables.

4. Conclusions

Response Surface Methodology is a useful tool to optimize and describe the extraction process using Hot Water Extraction. The water soluble β-D-glucan polysacharydes from baker yeast *Saccharomyces cerevisiae* were extracted using obtained parameters. An optimization for the two parameters was performed. The optimal theoretical extraction conditions were found to be temperature 100 °C and time 60 min. Under the optimal conditions the theoretical mass of polysaccharides was 376.1 mg, the actual average experimental value of extracted polysaccharides was 378.8 mg. The experimental and theoretical values are similar. These outcomes clearly indicate the adequacy of quadratic polynomial model to represent Hot Water Extraction of polysaccharides for two variables.

Acknowledgments

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Analytical Concentrating Systems Based on Polymeric, Inorganic, and Nanostructured Sorbents

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Keywords
analytical microsystems
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green chemistry
hydrocarbons

Abstract
The article is concerned with development of analytical microconcentrating systems and chromato-desorption type of sorption systems used for sample preparation of exhaled air. The systems are filled with polymeric, inorganic, and nanostructured sorbents. We have studied the particles' geometry and surfaces of the sorbents. The experimental estimation of sorption characteristics of the developed microconcentrating systems and optimization of the systems' geometry optimization has been carried out. It is shown that the microsystems filled with Al_2O_3 and carbon nanotubes (CNTs) are the most effective be-cause they allow to concentrate samples by 54 and 69 times, respectively. In the case of a sorption system, the concentrating coefficient for Al_2O_3 and MN-202 (commercial polymeric sorbent) are practically the same (429 and 480), but it is 3.2 times larger for CNTs (1433).

1. Introduction

It is necessary to concentrate the analyzed samples for determination of trace and ultratrace amount of substances in samples of exhaled air. This method is used in cases when the sensitivity of direct determination methods is not sufficient. Nowadays the solid phase microextraction (TFME) is widely spread method due to the trends of sample preparation devices' miniaturization, the refusal of organic solvents and the reduction of analysis stages due to the combination of sampling and sample preparation stages. The TFME method claims the place of the most suitable method for carrying out in-situ analysis and on-site analysis due to its peculiar properties [1, 2].

Of special interest are biomarkers, because it is possible to predict a functional disability of health due to change of their concentration in exhaled air. The object of the study has been the *n*-pentane which is the lipid peroxidation biomarker [3].

2. Experimental

Two types of analytical concentrating systems have been developed to solve the problem of quantitative determination of biomarkers in exhaled air. The first type of systems has been made of medical needles with 40 mm length and 0.5 mm inner diameter. Every system has been filled with sorbent manually. The second type of systems is glass syringe with length 74 mm and 8 mm inner diameter. We have chosen 3 types of sorbents: polymeric sorbent MN-202, inorganic sorbents Al_2O_3 and nanostructured sorbent CNTs.

The surface microstructures' analysis has been conducted with the use of scanning electronic microscope Tescan VEGA 3 SB. The image of Al_2O_3 particles has been made with zooming in $k = 70$, MN-202 particles $k = 80$, CNTs particles $k = 3000$. Larger zooming was needed to study surfaces of the sorbents: forAl_2O_3 $k = 3000$, for MN-202 $k = 2000$, for CNTs $k = 60010$.

Determination of the microsystems' sorption capacity has been carried out by means of passing of *n*-pentane:nitrogen mixture (C = 5 mg/m^3) through the thermostating system. The capacity of the systems up to slip has been calculated as area of the rectangle, one side of which is volume of the gas mixture up to slip and the second side is the initial concentration of gas mixture C = 5 mg/m^3. The total capacity of the system has been calculated as the area of the figure above the curve of sorption characteristic, which is limited from above by the line $C = 5$ mg/m^3. The model gas mixture has been prepared in a tedlar bag (SKC) using static volumetric method (when known volume of analyte is added in a container of known volume).

Concentrating of *n*-pentane from model gas mixtures with known concentration of the analyte has been conducted according to the scheme shown in Fig. 1. Sorption has been conducted at room temperature. We have passed model gas mixture through concentrating system and desorb analyte at high temperatures. Temperatures of desorption have been selected according to range of operating temperatures in a way to achieve the biggest amount of desorbed component.

Quantitative determination of analyte has been conducted by means of gas chromatography method under isothermal conditions with the use of the gas chromatograph Kristall 5000.1 (FID, HP-1 column).

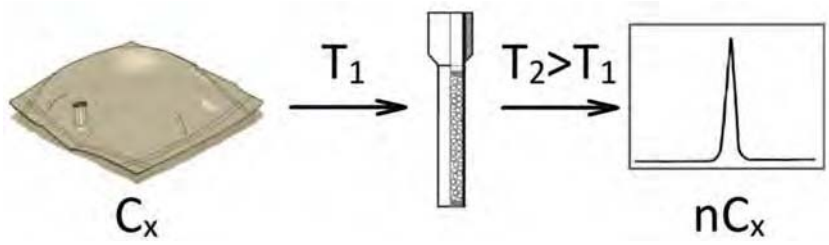

Fig. 1 Scheme of concentrating: (T_1) temperature of concentrating process, (T_2) temperature of desorption, (C_x) – analyte concentration in model gas mixture, (nC_x) analyte concentration after concentrating.

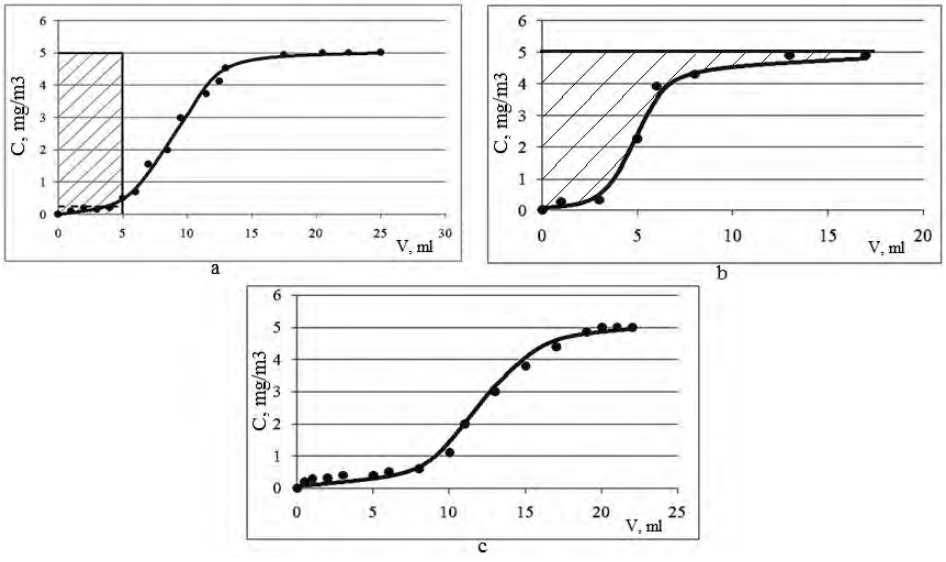

Fig. 2 Sorption curves of the studied microsystems (a) Al_2O_3, (b) MN-202, (c) CNT.

3. Results and discussion

Analysis of sorbents' surfaces has shown that particles of Al_2O_3 characterized by irregular geometric shape, and there are some smaller gains of sorbent with average size of 2.89 μm on the surface. So the sorbent obtains the additional sorption centers. There are macro- and micro pores on the surface of MN-202 sorbent. The CNTs has the most extended surface due to nanosized structure.

According to the images of sorbents we have calculated the average sizes of sorbents' particles. The average size of Al_2O_3 particles is 223.5 μm. The average size of MN-202 granules is 330.0 μm. The average diameter of fibers that contained CNTs is 3.5 μm, and the average diameter of CNT is 39.8 nm.

Fig. 2 shows the sorption curves. According to the sorption curves the volume up to slip for microsystem filled with Al_2O_3 is 5 ml, the total capacity is 13 ml of the model gas mixture. For microsystem filled with MN-202 the volume up to slip is 5 ml, the total capacity is 8 ml of the model gas mixture. For microsystem filled with CNT the volume up to slip is 8 ml, the total capacity is 17 ml of the model gas mixture.

According to the data it can be concluded that CNTs are the most promising for concentrating of analytes from exhaled air samples because of their ability to concentrate up to 69.4 μg on its surface. It is almost 9.5 times larger than the MN-202 capacity and practically in 15.5 times more than the Al_2O_3 capacity.

Sorbent MN-202 is also one of the most suitable for use, because it allows to sorb anlyte amount on each gram by 1.6 times more than Al_2O_3. But according to the fact that the weight of the MN-202 sorbent in the analytical microsystem is four times less than the weight of the other studied sorbents, the sorption capacity of the

Table 1
Results of concentrating (V_s – sample volume, T_d – desorption temperature, K – concentration factor).

Sorbent	V_s / ml	T_d / °C	K
System 1			
Al_2O_3	20	150	54
	10	200	12
MN-202	10	200	16
CNTs	20	200	69
System 2			
Al_2O_3	1000	200	429
MN-202	1000	200	480
CNTs	1000	200	1433

system generally is worse than the capacity of the microsystems filled with Al_2O_3. For the application of the MN-202 sorbent it is necessary to modify the system parameters, because the system is characterized by a large "dead" volume (about 70%) due to the geometric characteristics of the sorbent.

Table 1 shows the results of the concentration obtained carried out according to the scheme shown in Fig. 1. The concentrating coefficient K has been calculated as the ratio of the weight of *n*-pentane in the gas mixture after concentrating to the weight of *n*-pentane in the model gas mixture. The concentration of *n*-pentane in the model gas mixtures which was used for System 1 was 5 mg/m^3, and the gas mixture with *n*-pentane content of 0.25 mg/m^3 was used for System 2. According to the obtained data the most effective analytical microsystems (System 1) are the microsystems filled with Al_2O_3 and CNTs, they allow to concentrate the analyte by 54 and 69 times respectively. The concentrating coefficient for system 2 is K = 429.3 if we use Al_2O_3 sorbent, K = 480.4 if we use MN-202. The most effective sorbent is also CNTs, it is characterizes by concentrating coefficient K = 1433.5, which is 3.2 times larger than for other sorbents.

The obtained concentrating coefficient is enough to determine ppm amount of biomarkers in exhaled air samples, and by the means of modification of analytical microsystem parameters we can achieve concentrating coefficient which allow to determine ppb amount of analyte in exhaled air.

4. Conclusions

We have developed the analytical microsystems (System 1) and the sorption systems (Systems 2) for preparation of exhaled air sample. Systems have been filled with polymeric, inorganic and nanostructured sorbents. The properties of the sorbents and the developed systems have been studied. Particles' geometry and the sorbents' surfaces have been studied with the use of scanning electronic microscope. Image analysis allow to modify the configuration of the system and

increase the effectiveness of concentrating of trace and ultratrace amounts of an analyte. The experimental estimation of sorption characteristics of the developed microconcentrating systems has been carried out. It has been established that developed analytical microsystems allow to determine ppm amount of *n*-pentane in exhaled air samples.

Results of *n*-pentane concentrating from the model gas mixtures $C = 5$ mg/m^3 have shown the effectiveness of sorption system usage. Due to this fact experiments on concentrating of *n*-pentane from model gas mixtures with concentration $C = 0.25$ mg/m^3 have been conducted (the concentration is comparable to the content of the analyte in real samples). The most effective system is the system filled with CNTs, it is characterized by the largest concentrating coefficient $K = 1433.5$ among the studied sorbents. It should be noticed that application of analytical microsystems meets the requirements of green chemistry due to significant reduction of chemical agent and recourse usage, and in the case of thermal desorption, it completely eliminates the use of organic solvents.

Acknowledgments

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Development and Investigation of Gas Microsensors on a Chip Basis

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detector
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thermistor

Abstract
The gases concentration determination is significant and essential part of analytical chemistry. This research consists of the manufacture method and metrological properties analysis of thermochemical microsensors based on a chip. This article includes the parameters of magnetron sputtering, ultraviolet lithography, etching nanolayers of metals on a glass substrate for forming the sensing elements of the sensor. The technique of electrochemical polishing for forming a nanosized film of platinum on the working part of the thermocatalytic sensors. There were determined the planar resistors optimal geometry for forming the sensing elements of the sensor with a given resistance. We calculated the sensitivity of the fabricated sensor; as for methane it is 7.6×10^{-9} g/cm^3 and for hydrogen it is 8.3×10^{-10} g/cm^3.

1. Introduction

The main trend of analytical chemistry is the miniaturization of equipment with maintaining of the metrological characteristics of the larger counterparts. To reduce size and weight while increasing the speed, accuracy, and sensitivity of the analysis allows application in the design of microelectromechanical systems (MEMS) [1]. Mainly these systems are used for creating accurate accelerometers, gyroscopes, and sensors. The technology of creating sensors based on MEMS is time-consuming [2] and requires the use of special techniques, the application of operational layers of material on a substrate and forming the required geometry of the sensitive element (SE) detector [3]. There must consider the option of implementing these systems in already existing systems of analytical equipment, including gas chromatography (GC), to reduce the cost of industrial enterprises. This requirement involves the design and manufacture of housings of the detectors of the necessary size and forms, with the most efficient internal architecture to create a flow close to laminar [4].

All of above make relevant the fabrication and investigation of gas sensors for chip-based gas chromatography.

2. Experimental

2.1 The deposition of nanolayers of chromium and copper

Sensitive elements of gas sensors were obtained by ALD using equipment Caroline D12/A1. The growth of layers of chromium and copper were produced on glass substrates pre-heated to 200 °C. Thin films of chromium and copper were sputtered on the substrate, pre-cleaned with a concentrated sodium hydroxide. As a carrier gas and to purge the chamber used ultra-pure inert gas (99.999%), flow of which was 2 l/h. A film of the metals were deposited at a temperature of 120 °C, a pressure of 7×10^{-4} Pa and the speed of the drum 11 Rev/min. The time for deposition of nanolayers of chrome was 2 minutes for a nanolayer of copper 9 minutes. In this way was sputtered 20 nm of chromium and 200nm of copper. Control of the thickness of films was made by partial etching and measurement profilometer KLA-Tencor P-16+, scanning speed 10 μm/sec, the deviation from the required rate of <3%.

2.2 UV-lithography and etching

Formation of the necessary geometry of the sensitive elements of gas sensors took place in several stages. The first stage, through installation centrifugation Polos, uniformly deposited positive photoresist. Exposure samples were produced in the combining and exposure of EM-5006A with a UV illuminator using a specially made plastic template. The development process was carried out by washing the samples in the bath with a solution of potassium hydroxide in a few tens of seconds. Etching of copper was carried out with an aqueous solution of iron chloride. Next, the samples were washed with distilled water and dried with an air gun. The chromium etching was carried out using a solution of cerium sulfate and sulfuric acid. Washing was done with distilled water, followed by drying by the air gun. The process of removing the residual photoresist was carried out with concen-trated sodium hydroxide. Washing was done with distilled water, followed by drying by the air gun.

2.3 Electrolytic polishing, and a schematic circuit of the geometry of microsensors on chip-based

To increase the sensitivity of the gas microsensors was carried out by forming the catalyst layer on one of the paths from the sensitive element. The process of applying the catalytic platinum layer on the planar resistor is shown in Fig. 1 and represents the electric current is passed through a freshly prepared electrolyte solution consisting of a solution of platinochloride acid.

Were measured resistivity paths with a catalytic layer and without it. By the obtained parameter values were generated schematic circuit of the probe gas microsensors on the chip basis, as shown in Fig. 2. Fig. 3 shows the final view of the gas microsensors on chip-based.

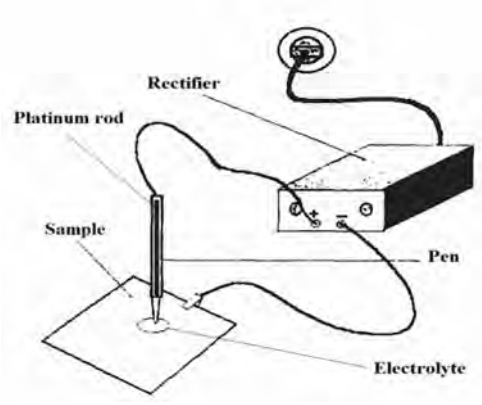

Fig. 1 The schematic circuit of electrolytic polishing

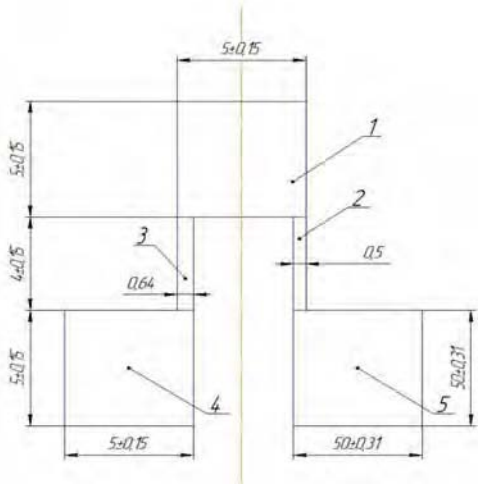

Fig. 2 The schematic circuit of gas microsensors on chip-based: (1, 4, 5) pressure contacts, (2) microsensors with a catalytic layer of platinum, (3) microsensors without catalytic layer of platinum.

2.4 The housing of the detector to the microsensors on chip-based

To ensure optimal supply and discharge of gas was designed and with the help of milling machine formed the housing of the sensor with the effective geometry of the channels. To facilitate the conditions of the experiment detector housings were made detachable, consisting of top and bottom parts. 3-D models of the housing and its components are shown in Fig. 4 (on the next page).

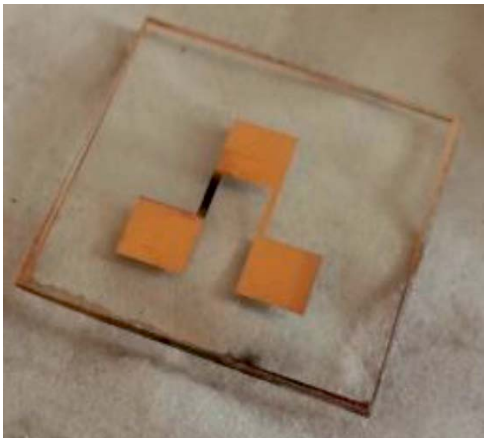

Fig. 3 The sensing element of the gas microsensors on chip-based .

2.5 Determination of the main metrological characteristics of gas microsensors in the portable microchromatograph system PIA

Samples of sensitive elements of gas microsensors on a chip base were installed in a projected detector body. This detector is included in the microfluidic gas microchromatograph system PIA.

To calculate the minimum amount of analyte, which can be determined with the using of a gas microchromatograph PIA, the detection limit of the

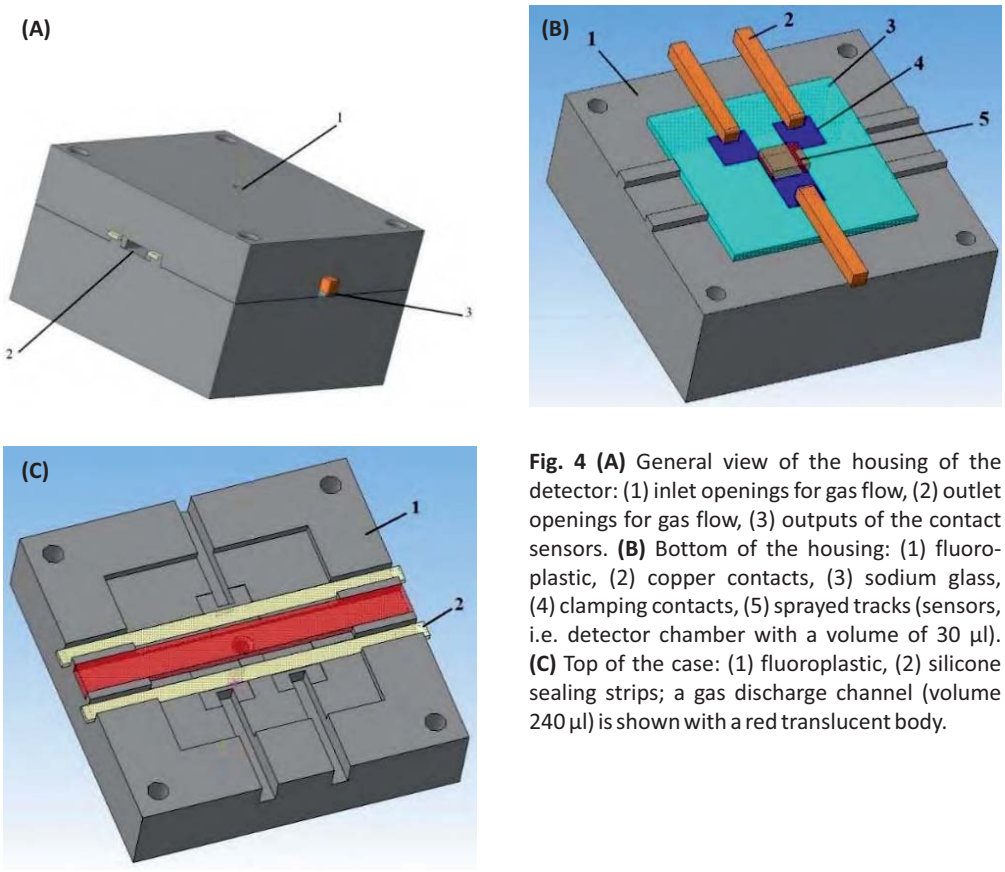

Fig. 4 (A) General view of the housing of the detector: (1) inlet openings for gas flow, (2) outlet openings for gas flow, (3) outputs of the contact sensors. **(B)** Bottom of the housing: (1) fluoroplastic, (2) copper contacts, (3) sodium glass, (4) clamping contacts, (5) sprayed tracks (sensors, i.e. detector chamber with a volume of 30 µl). **(C)** Top of the case: (1) fluoroplastic, (2) silicone sealing strips; a gas discharge channel (volume 240 µl) is shown with a red translucent body.

thermochemical detector was determined. The detection limit of the thermochemical detector C_{min} [g/cm^3] was calculated by the formula

$$C_{\text{min}} = \frac{2\,\Delta_x\, C_{\text{CGS}}\, V_{\text{PET}}}{\overline{S}\, F} \tag{1}$$

where Δ_x is the maximum value of the amplitude of repeated oscillations of the zero signal with half-period (pulse duration) not exceeding 10 s, C_{CGS} the concentration of the control component, i.e. calibration gas mixture [mg/m^3], V_{PET} the volume of the dosing singer [ml], $\overline{S}$ the arithmetic mean of the peak area [V s], and F is the consumption of carrier gas [ml/s].

The relative standard deviation (*RMS*) was calculated by the formula

$$RMS = \frac{1}{C_{\text{meas}}} \sqrt{\frac{\sum_{i=1}^{n} (C_{\text{meas},i} - C_{\text{meas}})}{n-1}} \cdot 100\% \tag{2}$$

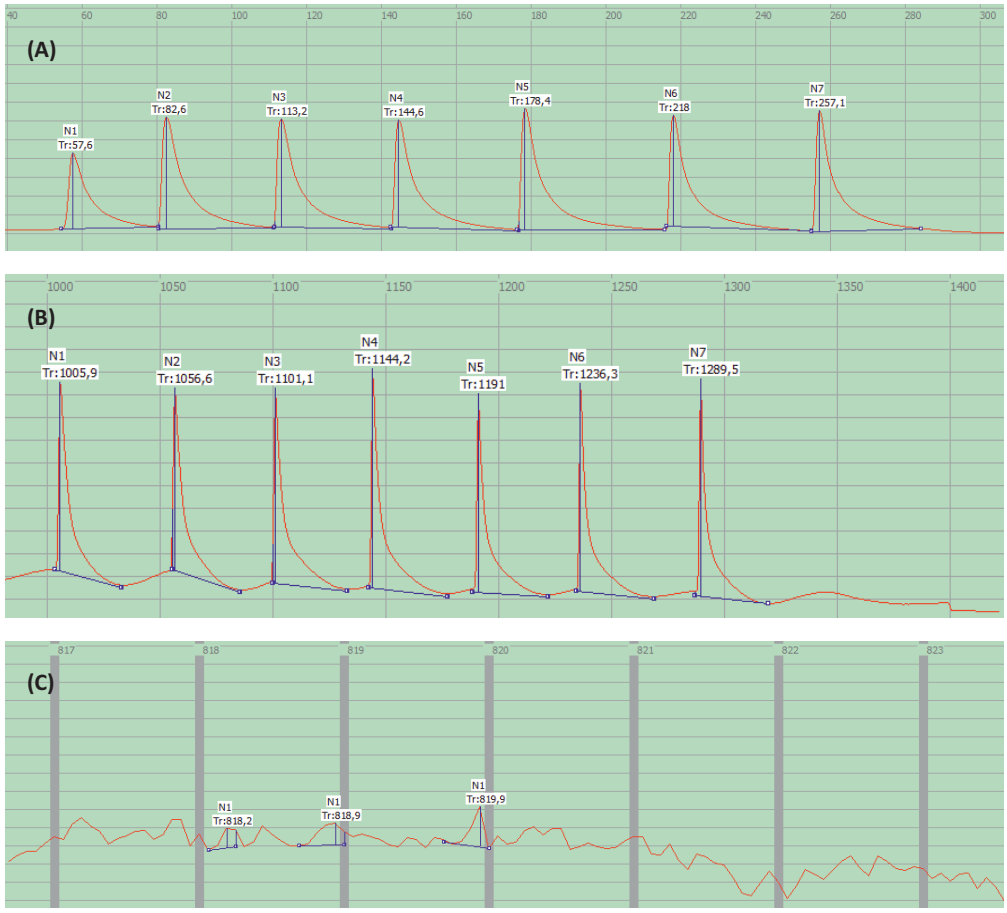

Fig. 5 (A) Chromatogram of methane. (B) Chromatogram of hydrogen. (C) Noises on the chromatogram.

where n is the number of values in the sample, $C_{\text{meas},i}$ the measured value of the i-th component, and C_{meas} the average measured value.

3. Results and discussion

To study the dependence of the resistance of the sensitive elements on the temperature, the samples were subjected to heating from 20 to 200 °C. It was found that the dependence of film resistance on temperature on the average values of four samples of sensitive elements of gas microsensors is linear in the measured range.

The main metrological characteristics were determined by connecting a gas microsensor enclosed inside the formed detector body to the PIA gas microchromatograph detection system. The experiment included the determination of the response signal of the sensor on the calibration gas mixtures of methane and hydrogen in the carrier gas stream. Air was used as carrier gas. The concentration

of the substances in the air stream was 30%, the volume of the sample was 10 ml, the flow rate was 100 ml/min, the current was 100 mA, and the thermostat temperature was 150°C.

According to the chromatogram (Fig. 5A), the main values of chromatographic peaks are determined and, according to equation (2) *RMS* is calculated for the area and height, which is not used by 3%.

Calculations were made from the chromatogram of hydrogen (Fig. 5B) in the air flow, with the same experimental parameters, for which it was found that the *RMS* also does not exceed 3%.

The values of the noise height of the gas microsensors (Fig. 5C) required for further calculations.

According to the chromatogram, the sensitivity of a gas sensor on a chip base is determined, which is 7.6×10^{-3} g/m^3 (11.4 ppm) for methane and 8.3×10^{-4} g/m^3 for hydrogen (19.8 ppm).

4. Conclusions

The result of this work has developed a method of making gas sensors for chip-based gas chromatography. The effective value of the thickness of a chromium film on a glass substrate was revealed, for the best sensitivity in the temperature range from 20 to 200 ° C, which was 20 nm. All samples of chromium films have a linear dependence in this temperature range. Further heating leads to a break-down in the contact group. The process of depositing a catalytic layer of platinum using an electrolyte solution, with a flowing current of the small force, has been developed. During the experiment, the proper application time is revealed, after which the copper layer begins to bleed off with a sharp increase in the resistance of the planar resistor. Designed and manufactured a casing for a gas microsensor on a chip base with an efficient geometry of the gas supply and removal. The main metrological characteristics of the obtained gas microsensors on a chip basis are determined in an experiment with verification gas mixtures of methane and hydrogen.

Acknowledgments

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A Large-Surface Carbon Film Electrode Used for Voltammetric Determination of Imipramine Hydrochloride

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Keywords
imipramine hydrochloride
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voltammetry

Abstract
A large-surface carbon film electrode (ls-CFE) was used for voltammetric investigation of an antidepressant drug imipramine hydrochloride using differential pulse voltammetry (DPV) and cyclic voltammetry (CV). Under the optimal conditions found, imipramine hydrochloride was determined in buffered aqueous solutions in the concentration range from 0.1 to 100 μmol L^{-1} by DPV. Furthermore, the applicability of the newly developed DPV method was verified on model samples of drinking water.

1. Introduction

Imipramine hydrochloride (Fig. 1) is a tricyclic antidepressant of the dibenzazepine drug family. It was discovered in 1951 [1] and it is primarily used to treat depression [2, 3] (associated with anhedonia [4]) and nocturnal enuresis [5]. The imipramine hydrochloride undergoes the electrochemical oxidation on the nitrogen containing ring [6–8]. Bishop and Hussein [8] reported that one electron is removed from the nitrogen atom and a cation radical is formed which can exist in a number of resonance forms. This monocation dimerises or reacts with an unoxidised molecule. The dimerisation is accompanied by the loss of two protons per dimer. The dimer is more easily oxidised than the monomer and is forming a dication with the loss of two electrons per dimer. [9]. This enables to use voltammetric techniques in the anodic region of potentials for its determination.

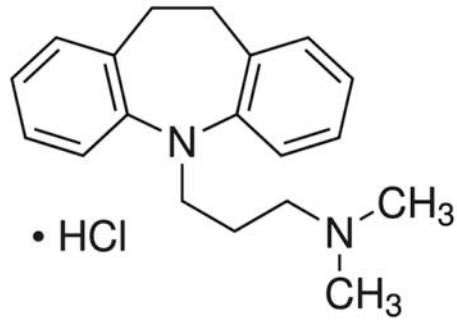

Fig. 1 Structural formula of imipramine hydrochloride.

A large-surface carbon film electrode [10, 11] (ls-CFE) was used in this study as a working electrode for the sensitive voltammetric determination of imipramine hydrochloride. The surface of the working electrode is covered with black ink film based on a microcrystalline graphite–polystyrene composite. The composite film can be applied on the surface of any solid electrode (e.g., Pt, Au, Ag) and can be easily mechanically removed before each measurement, thus eliminating the passivation of the electrode surface [11–13]. Additional advantages of the ls-CFE are also its wide potential window, good reproducibility of the film preparation, elimination of problems associated with the "history of the electrode", and simple chemical modification of the electrode surface [14].

2. Experimental

2.1 Reagents and chemicals

All used chemicals were of p.a. purity. A stock solution of imipramine hydrochloride (99%, Sigma-Aldrich, Germany) (c = 1×10^{-3} mol L^{-1}) was prepared in deionized water (Millipore Milli-Q Plus system, Millipore, USA). Dilute solutions were prepared by exact dilution of the stock solution with deionized water. The stock solution was stored in dark and in refrigerator. Britton-Robinson (BR) buffer solutions (c = 0.04 mol L^{-1}) were prepared in a usual way [15] and used as supporting electrolytes. The substrate gold electrode (PEEK electrode body diameter of 7 mm, gold disk diameter of 2 mm, Metrohm, Switzerland) was modified by 10 µL of a carbon ink prepared by mixing 0.01 g of polystyrene, 0.09 g of microcrystalline graphite (particle size of 3.5–5.5 µm, Graphite Týn, Czech Republic), and 0.5 mL of 1,2-dichloroethane (99.97%, Merck, Germany). All chemicals were used without further purification and their solutions were kept in glass vessels in dark at laboratory temperature.

2.2 Instrumentation

An Eco-Tribo Polarograph with PolarPro 5.1 software (Polaro-Sensors, Czech Republic) was used for all voltammetric experiments. A three-electrode arrangement was used in individual measurements: a silver|silver chloride reference electrode (type 10-20+polaro, 3 mol L^{-1} KCl), a platinum wire auxiliary electrode (type Pt 1+polaro) (both Monokrystaly, Czech Republic), and the ls-CFE working electrode were used. Experimental parameters for DPV were: a pulse width of 100 ms, a pulse modulation amplitude of 50 mV, a sampling time interval of 20 ms, and a scan rate of 20 mV s^{-1}. In CV, the scan rates varied from 5 to 500 mV s^{-1}. A Lab Dancer (IKA, China) vortex was used for mixing the carbon ink. Spectrophotometric measurements were realized at HP 8453 Diode Array Spectrophotometer (Hewlett-Packard, Netherlands) with operating program UV-Visible ChemStation (ver. 9.01) using 0.1 cm quartz cuvettes. Digital pH-meter Jenway 3510 with

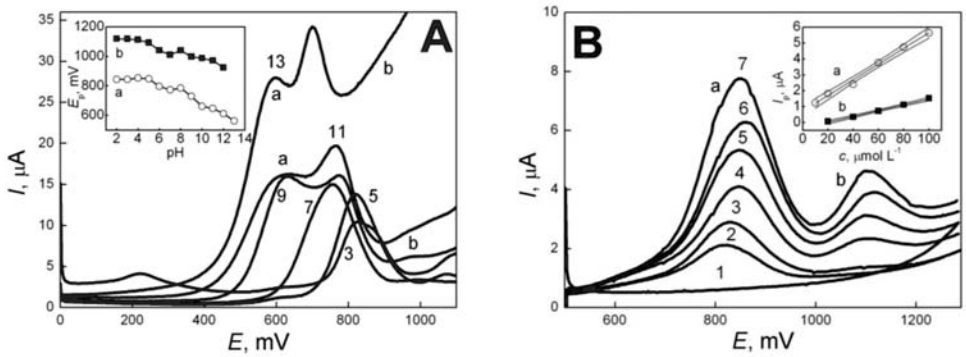

Fig. 2 (A) Voltammograms of imipramine hydrochloride ($c = 1\times10^{-4}$ mol L^{-1}) registered using DPV at the ls-CFE in BR buffers; depicted are the voltammograms obtained at the newly prepared ls-CFE when measuring the first scan at odd pH values of BR buffer: 3.0, 5.0, 7.0, 9.0, 11.0, and 13.0. Inset: dependence of the peak (*a* and *b*) potential (E_p) on pH. **(B)** Voltammograms of imipramine hydrochloride registered using DPV at the ls-CFE in a sample of deionised water:BR buffer pH = 4.0 (9:1) in the concentration range of 10–100 μmol L^{-1}; concentration of imipramine hydrochloride: (1) 0, (2) 10, (3) 20, (4) 40, (5) 60, (6) 80, and (7) 100 μmol L^{-1}. Inset: corresponding calibration curves for peaks *a* and *b*; the confidence bands are constructed for α = 0.05 (*n* = 4).

combined glassy electrode (type 924 005) (both Jenway, UK) was used for pH measurements. The instrument was calibrated using standard aqueous calibration buffers.

3. Results and discussion

Firstly, the optimal medium pH for the imipramine hydrochloride determination using DPV at the ls-CFE was sought (Fig. 2A). The BR buffer solutions in the pH range from 2.0 to 13.0 were investigated. Imipramine hydrochloride (c = = 1×10^{-4} mol L^{-1}) gives one well developed peak in the whole pH range and a second lower peak in the pH range from 2.0 to 12.0. The second lower peak was not observed at pH = 13.0. Each measurement at different pH was performed on a new film. Only the first scans are depicted in Fig. 2A. Second scans on each film provide another peak at lower potentials (E_p around 200 mV and lower). This seems to be due to the passivation of the electrode surface by oxidation products. BR buffer pH = 4.0 was chosen as the optimal medium.

Secondly, the repeatability of the measurement (20 consecutive scans) on individual carbon films (5 films) was tested using DPV of 1×10^{-4} mol L^{-1} imipramine hydrochloride in BR buffers pH = 2.0, 4.0, and 7.0. There was a gradual decrease in the peak current observed probably due to electrode passivation [9]. Therefore, the repeatability of the formation of individual carbon films was tested under the optimal conditions found (BR buffer pH = 4.0), with the obtained relative standard deviation (RSD) values of 30% for the potential window from 0 to 1400 mV, and 6% for the potential window from 500 to 1300 mV. Therefore,

the first curve and shorter potential window were always used to construct the calibration plot and all measurements were performed at a fresh carbon film surface. The found optimal conditions were used to construct calibration dependences of imipramine hydrochloride (Fig. 2B), which were linear in the concentration range from 0.1 to 100 μmol L^{-1}, with the limit of quantification ($LOQ = 10\sigma/\text{slope}$) of 3×10^{-7} mol L^{-1}.

The applicability of the newly developed voltammetric method was verified on model samples of drinking water (obtained from public tap in the building of Institute of Chemistry, Faculty of Science, Charles University, Prague). Measured solutions consisted of 9.0 mL of a drinking water model sample and 1.0 mL of BR buffer pH = 4.0 to adjust pH. Small standard additions of the imipramine hydrochloride stock solution were spiked into the samples to construct calibration curves. The limit of quantification (LOQ) was 2×10^{-7} mol L^{-1} for drinking water.

Thirdly, CV at the ls-CFE was used to examine the influence of the scan rate to demonstrate which processes are controlling the electrochemical oxidation of imipramine hydrochloride, which is a quasireversible process at the ls-CFE. The non-linear dependence registered in both cases (peak current (I_p) vs. square root of the scan rate, and I_p vs. scan rate) demonstrates that the electrode process is under mixed control of both diffusion and adsorption.

4. Conclusions

Differential pulse voltammetry (DPV) and cyclic voltammetry (CV) were used in the study of voltammetric behaviour of the drug imipramine hydrochloride. Optimized conditions were investigated for the determination of the studied analyte at the large-surface carbon film electrode (ls-CFE) and the newly developed method was verified on model samples of drinking water. The information about the electrochemical oxidation of the studied drug and about its reversibility was obtained using CV. The electrochemical reaction is under the mixed control of both diffusion and adsorption.

Acknowledgments

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Nonlinear Standard Addition Method in Determination of Paracetamol

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Keywords
analytical calibration
flow analysis
paracetamol
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spectrophotometry

Abstract
The main object of this paper is to present how adjustment of calibration curves to registered measurement points affects accuracy and precision of the obtained analytical results in determination of paracetamol in pharmaceuticals with the use of the standard addition method in flow-injection technique. The employed method was based on nitrification of paracetamol in reaction with sodium nitrate in acidic environment of hydrochloric acid of different concentrations. The formed derivative species reacted further with sodium hydroxide to convert it into a more stable compound for which absorbance was recorded at the wavelength of 430 nm. The use of the polynomial standard addition method allowed compensation of an existing multiplicative interference effect. This approach enabled improvement of accuracy of the obtained results in the case of all analysed pharmaceuticals. The proposed procedure, due to ease of implementation and short time of analysis combined with low reagent consumption, fully meets the requirements of the so-called "green analytical chemistry".

1. Introduction

Paracetamol, also known as acetaminophen, is one of the most popular painkillers and antipyretics. Incorrectly belongs to the group of non-steroidal anti-inflammatory drugs due to its antipyretic and analgesic effect, but compared to this group of drugs, it has no anti-inflammatory effect. It also shows a different mechanism of action than non-steroidal anti-inflammatory drugs [1]. It is selective inhibition of COX-3 cyclooxygenase in the central nervous system and the impact on the thermoregulation center which is responsible for its analgesic and the antipyretic effect. It also affects the serotoninergic system, causing pain relief [2]. If you use pharmaceuticals containing paracetamol at the maximum therapeutic dose, there are no serious side effects except for likely allergic skin reactions. The advantages of using paracetamol are: wide therapeutic range, good tolerance by the human body, very good bioavailability, fast elimination from the body and low toxicity. Abuse and long-term consumption of paracetamol can lead

to impaired renal function, increased blood pressure, liver damage or greater likelihood of myocardial infarction [1]. There are several methods of paracetamol determination based on the use of such analytical techniques as UV-Vis spectrometry, infrared spectrometry, cyclic voltammetry, amperometry, high performance liquid chromatography, gas chromatography, or capillary electrophoresis [3].

In the case when an interference effect is present in the analyzed samples and when the preliminary step of the analytical procedure does not ensure its elimination, the use of a proper calibration method can lead to the final result affected by a systematic error. An example of such a method is the standard addition method (SAM), which provides compensation for the multiplicative interference effect [4]. In the case where the measurement points show a slight deviation from a straight line application of a linear approximation to plot a calibration graphwill likely lead to resultsaffected by a systematic error. This presentation shows how inappropriate calibration fitting can influence the obtained results on the example of paracetamol determination using flow technique with the standard addition technique.

2. Experimental

2.1 Reagents and samples

The following reagents were used: paracetamol (Sigma-Aldrich, Germany), sodium nitrite (POCH, Poland), hydroxide sodium (POCH), concentrated hydrochloric acid (Merck, Germany), and E110 food coloring (Hokus, Poland).

Paracetamol stock solution (5 g L^{-1}) was prepared by dissolving 0.5 g of paracetamol in 100 mL distilled water. The solution served for preparation of a synthetic sample of 100 mg L^{-1} and a set of standard solutions. A solution of hydrochloric acid at the concentration of 0.01 mol L^{-1}, 0.1 mol L^{-1}, 0.2 mol L^{-1} was prepared by taking 0.41 mL, 4.15mL, 8.3 mL 35% hydrochloric acid, respectively, into a volumetric flask of 500 mL and filling with demineralised water to the mark. 0.14 mol L^{-1} sodium nitrite solution was prepared by dissolving 4.83 g of sodium nitrate in 500 mL of distilled water. 0.1 mol L^{-1} sodium hydroxide solution was prepared by dissolving 4 g of sodium hydroxide in 1000 mL of distilled water. A solution of 1.2 g L^{-1} food coloring solution was prepared by dissolving 60 mg of E110 dye in 1.25 mL of ethanol in a 50 mL flask and filling with 0.1 mol L^{-1} hydrochloric acid to the mark. A synthetic sample of 100 mg L^{-1} paracetamol with E110 dye was prepared by mixing 0.5 mL of 5 g L^{-1} paracetamol solution and 0.5 mL of 1.2 g L^{-1} dye in a 25 mL flask and filling it to the mark with 0.1 mol L^{-1} hydrochloric acid solution. Distilled water from an HLP 5 system (Hydrolab, Poland) was used throughout the work.

The following samples were tested: Theraflu ExtraGrip (GlaxoSmithKline Consumer Healthcare), Vicks SymptoMed Complete (Teva Pharmaceuticals Poland, Febrisan (Takeda Poland).

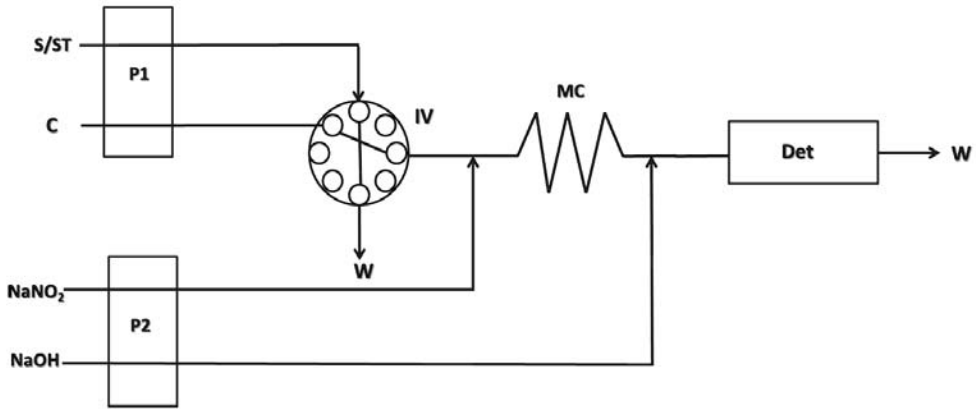

Fig. 1 Scheme of the employed flow-injection system: (C) carrier stream, (ST) standard solution, (S) sample solution, (P1, P2) peristaltic pumps, (IV) injection valve, (MC) mixing coil, (W) waste, (Det) detector.

2.2 Instrumentation

A flow-injection system shown in Fig. 1 was used for determination of paracetamol. It consisted of two Minipuls 3 peristaltic pumps (Gilson, France) and an injection valve (Perkin Elmer, USA) equipped with a control system made in our laboratory. Lambda 25 spectrometer (PerkinElmer, USA) equipped with a 10 mm flow cell was used as the detector. Additionally, a 16-channel controller UVCTR-16 (KSP Elektronika Laboratoryjna, Poland) with Valve and Pump Controller software (KSP Electronics Laboratory, Poland) were used to control pumps and the valve. Sonic-3 ultrasonic bath (Polsonic, Poland) was used to remove gases from solutions.

The samples were analysed with the use of the designed and constructed flow system. A sample/standard solution was injected into a stream of a carrier, i.e. a solution of hydrochloric acid which was connected with a stream of sodium nitrate(III) solution, resulting in formation a nitroso derivative of the analyte. The formed product was subsequently stabilized with sodium hydroxide solution. The yellow reaction product was directed towards the detector where absorbance was recorded at the wavelength of 430 nm. Three different concentrations of hydrochloric acid, i.e. 0.01, 0.10 and 0.20 mol L^{-1}, were used to differentiate the reaction conditions and to obtain three calibration curves of different sensitivity. Height of the recorded characteristic peak with a plateau area was treated as the analytical signal. Each determination was repeated three times in the same experimental conditions.

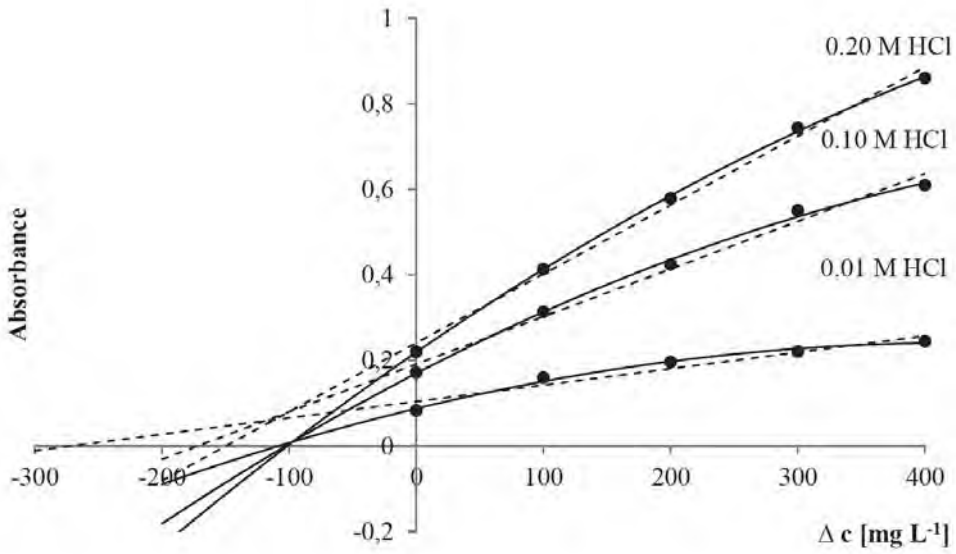

Fig. 2 Set of calibration curves created using a linear (dotted line) and polynomial (continuous line) plot obtained for the sample of 100 mg L^{-1} paracetamol

3. Results and discussion

Optimization studies were conducted on the basis of the synthetic sample (100 mg L^{-1} of paracetamol). The following parameters were chosen: injection loop 70 μL; flow rate ratio r_1/r_2 = 1.0; mixing coil 100 cm; flow rates 2.0 mL min^{-1}, and single peak time 100 s.

In the presented studies the SAM was used with various functions appropriately fitted to experimental results: linear and polynomial of degree 2. This operation was designed to show how inappropriate approximation could lead to a result affected by a systematic error. Fig. 2 presents polynomial calibration curves obtained for a synthetic sample with 4 standard additions and in three hydrochloric acid concentrations, which provided accurate results.

If one of the three graphs is constructed with lower accuracy than the other two, it is possible to verify the results and to minimize the risk of receiving an inaccurate result. Based on the obtained results, we decided to use a nonlinear approximation owing to higher precision and accuracy of the obtained results. Fig. 3 (on the next page) presents the calibration curves for Theraflu sample with four standard additions. By comparing results obtained using linear and polynomial approximation, overestimated results are obtained with a linear fitting. It can be seen that in this case precision of the results is much worse than with a nonlinear approximation.

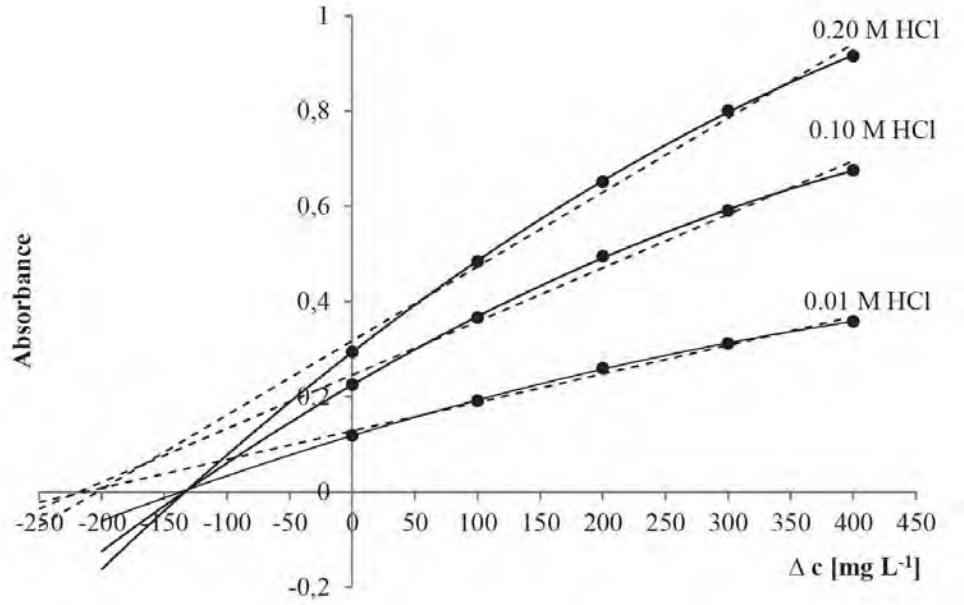

Fig. 3 Set of calibration curves generated for Theraflu sample.

Table 1
Final results obtained for syntethic and real samples using linear and non-linear approximation ways.

Sample	Expected concentration / mg L^{-1}	Found concentration / mg L^{-1}			
		Linear Approximation	*RE* (%)	Non-linear Approximation	*RE* (%)
Synthetic	100.00	200.07 ± 114.56	100.07	103.88 ± 6.48	3.88
Synthetic with dye	100.00	615.84 ± 646.80	515.84	344.55 ± 193.15	244.55
Febrisan	75.00	121.59 ± 15.52	62.12	82.61 ± 2.83	10.15
Vicks	50.00	82.29 ± 1.85	64.58	51.91 ± 14.48	3.83
Theraflu	65.00	105.72 ± 2.13	62.65	67.24 ± 15.66	3.44

Table 1 collects results obtained for synthetic sample, synthetic sample with a dye and three real sample: Febrisan, Vicks and Theraflu. The results obtained using a linear approximation are worse in precision and accuracy than those obtained using a nonlinear approximation. In the case of the synthetic sample with a dye the use of the SAM method both for linear and polynomial approximation could not compensate the additive effect caused by color of the additive, which is a serious limitation in routine assays. The final results were affected with a significant systematic error.

In the case of real samples analyzed using a nonlinear second-degree fitting, comparison of the results obtained with the proposed method with paracetamol

content given by the manufacturers revealed good accuracy (the relative error for most tested samples were not higher than 4%) and high precision of the proposed method. The error caused by the color of the real samples was not observed here, since the samples were diluted 100 times and their color no longer caused the additive interference effect.

4. Conclusions

The constructed flow-injection system operated properly and repetitively. Application of a nonlinear approximation enables to avoid overstatement of results in comparison with a linear regression. The results obtained with the use of polynomial curves were characterised by very good accuracy It should be noted here that the experimental results presented above are an excellent example of what linear approximation for calibration dependencies with even very slight deviations from the straight line can lead to. In laboratory practice it is very rare to draw attention to such small deviations as in Fig. 1. If in the interpolation method, linear approximation does not have a greater significance to the value of the obtained result, in the extrapolation methods even the slightest deviations from the linearity have very serious consequences.

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Using Chromato-Desorption Microsystems For Determination Breath Acetone Concentration

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Keywords
biomarkers
breath acetone
chromato-desorption microsystems
diabetes
non-invasive diagnostics

Abstract
The results of developing methods increasing accuracy of biomarker quantitative determination in exhaled air are shown in this research work, it has been achieved by using the injection type of chromato-desorption microsystems. We have made the comparison of offered method and standard methods. It has shown the expediency of using chromato-desorption microsystems.

1. Introduction

According to official statistics of the World Health Organization [1], the number of people with diabetes increased to 422 million. It is known that diabetes can lead to various medical complications, such as kidney failure, amputation of limbs, blindness, heart attack and stroke, so early diagnosis of this disease is an urgent problem.

To diagnose and monitor diabetes routine are used laboratory tests: detection of high blood sugar (hyperglycemia) and urine (glucosuria). The disadvantage of known methods is laboriousness and invasiveness, it leads to the patient physical and emotional discomfort. In connection with this, one of the most acute problems of modern medicine is the development of non-invasive methods for determining the level of glucose in the blood, necessary for patients with diabetes mellitus.

Currently, the composition of exhaled air and the possibility of using its analysis for early diagnosis and monitoring of diseases are being actively studied, which will reduce the cost of medical diagnostics and improve the quality of life of patients. Exhaled air is a multicomponent gas mixture in which more than 800 chemical compounds are present. About 20 of them are most sensitive to changes in functional health, they can be predictors of some diseases. For example, studies

[2, 3] note that the concentration of acetone in the exhaled air is related to the level of glucose in the blood, which makes it possible to use acetone as a biomarker of diabetes mellitus. The development of non-invasive diagnostics is hampered by the lack of an optimized method for the quantitative determination of the microquantities of acetone in the exhaled air. One of the limiting factors determining the accuracy and speed of biomarker measurements in exhaled air is sampling and sample preparation, which exclude the introduction of additional impurities.

The purpose of the research is the development of methodological techniques and the quantitative determination of acetone in exhaled air tools.

2. Experimental

2.1 Reagents and chemicals

We have chosen three types of sorbents for micro systems Chromaton N-AW--MCS+25% $CaCl_2$, Al_2O_3, and MN-202 and filled with them developed chromato-desorption microsystems (ChDmSs). We have used acetone 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 0.5mm) and filled with sorbents. Fig. 1 shows a schematic diagram ChDmS and a photo of the finished device.

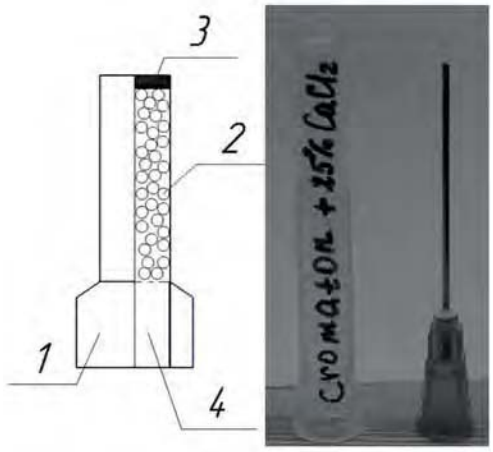

Fig. 1 **(a)** The schematic diagram of micro ChDmS, and **(b)** photo of fabricated device (1) needle hook, (2) sorbent, (3) plug, (4) internal channel of needle.

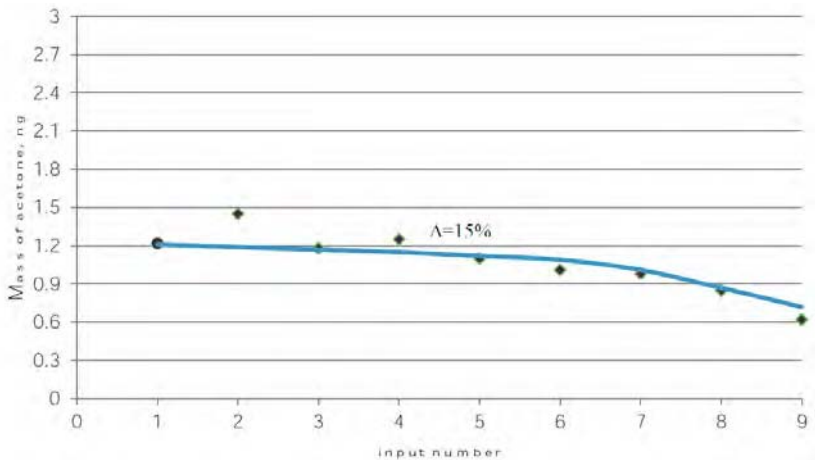

Fig. 2 Acetone concentration in dependence on ChDmS filled Cromaton N-AW-DMCS + 25% $CaCl_2$ sorbent usage period under conditions of discrete dosing.

3. Results and discussion

It is known that surface-layer sorbents, modified with sorption-active inorganic salts, have a large adsorption capacity, chemical inactivity and thermal stability, allow the process of concentrating a sample with direct thermal desorption of impurities to transfer them to a gas chromatograph, thereby shortening the time, increasing the sensitivity Analysis . In this connection, it is expedient to study the potential use of sorbents of this type for the manufacture of chromatographic desorption microsystems designed to produce gas mixtures containing microquantities of acetone.

The polymer sorbent MN-202 is characterized by macro and micro pores presence and 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. Chromaton N-AW-DMCS with modification of 25% $CaCl_2$ 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. The cumulative effect of these factors makes it possible to predict an increase in sorption capacity of the system. A similar effect of increasing the sorptive capacity of the system due to the large surface area and is shown to Al_2O_3, the capacity reduction is not observed.

It was established experimentally that ChDmS life of dispensing discrete gas mixture of at least six cycles with a standard deviation δ =15% (Fig. 2).

4. Conclusions

Chromato-desorption microsystems have been developed. Standard methods and methods for the determination of volatile organic compounds by the example of acetone in air environment experimentally has been researched and microanalytical chromatographic-desorption systems has been developed. It is established that when discrete dosing of a gas mixture containing a standardized amount of acetone, the operating life of ChDmSs is at least six cycles. Wherein the renewable resource of the system is at least four cycles. Application of the developed microanalytical systems has several advantages, the main of which are the simplicity of hardware design, universality, efficiency, exponentiality and the possibility of automation of analysis.

Acknowledgments

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Characterization of Nutritional Supplement With Content of *Vaccinium macrocarpon* Based on Comparison of Chosen Flavonol Glycosides by HTGC-MS 126

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Keywords
flavonol glycosides
high temperature gas chromatography
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Abstract
High temperature gas chromatography with mass detector was used for separation and determination of high molecular weight compounds: glycosidized flavonols. This method was used for determination of glycoside derivates of quercetin in the form of quercetin 3-O-β-D-galactoside, quercetin 3-O-β-D-glucoside, and quercetin 3-O-α-D-arabinoside. After two-step derivatisation process, selected flavonol glycosides derivates were identified in four samples of a nutritional supplement containing cranberry extract, cranberry jam and three samples of the dried berries. The determined values of the total content of the investigated substances in the samples were significantly different and are in range of 5–16400 mg kg^{-1}.

1. Introduction

Flavonoids are substances which are included into the group of so-called antioxidants [1, 2]. At present a high number of positive effects on human health of these phenolic compounds are known. They have significant anti-inflammatory, antitumor, antiviral and antibacterial effects [3, 4]. Due to their antioxidant activity, flavonoids have found application in the pharmaceutical industry and are used in many forms of nutritional supplements such as tablets, capsules or syrups. In the manufacturing of nutritional supplements are used extracts from plants which are incorporated into the resulting pharmaceutical form [5] by compression or by other processes. The most common natural source of flavonoids is American cranberry (*Vaccinium macrocarpon*) [6]. Together with cranberry blueberry (*Vaccinium myrtillus*), they are a significant source of glycoside

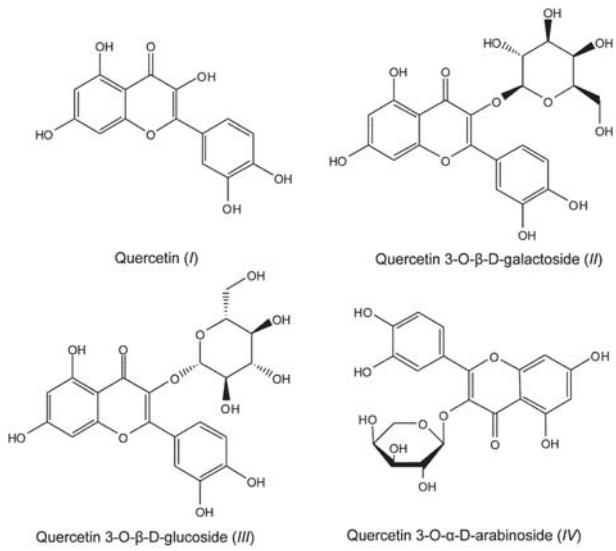

Fig. 1 Structural frormulas of quercetin and its selected glycosides derivates.

derivates of quercetin. For example in Fig. 1 are shown several types of flavonol glycosides of quercetin. As members of the flavonoids specie, flavonols themselves do not have a large molecule, due to which their identification and quantification by gas chromatography is relatively simple. When a glycoside derivate is formed, the hydrogen atom in one hydroxyl group is replaced by one carbohydrate unit and this produces a molecule so large that it cannot be detected by commonly used gas chromatographic methods. For this reason, HPLC methods are more commonly used for determination of these compounds [7]. Nevertheless, it is possible to use high-temperature gas chromatography with a mass detector (HTGC-MS) which, after appropriate derivatisation, allows determination of such large molecules [8, 9].

The aim of this work is the development of a new method using high-temperature gas chromatography for identification and quantification of selected flavonol in nutritional supplements with extracts from American cranberry and its dried berries.

2. Experimental

2.1 Reagents and chemicals

For analysis, four types of nutrition supplements with *Vaccinium macrocarpon* extract were used in various dosage forms: Sample 1 (dosage form: tablet), Sample 2 (dosage form: tablet), Sample 3 (dosage form: capsule), Sample 4 (dosage form: tablet). Samples were purchased from local pharmacies. Three types of dried cranberries (Cranberries 1–3) and cranberry jam (Cranberry jam)

were purchased on the market. The derivatisation agent 1,1,1,3,3,3-hexamethyldisilazane (HMDS) and acetonitrile were purchased from Merck (Germany), trifluoroacetic acid and *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Fluorochem (Great Britain). Quercetin 3-O-β-D-galactoside, quercetin 3-O-β-D--glucoside, quercetin 3-O-α-D-arabinoside, from Sigma-Aldrich (Germany) was used as the standard.

2.2 Instrumentation

Ultrasonic bath (Kraintek, Slovakia) and gas chromatograph Network GC System 6890N (Agilent Technologies) with MS detector (5973N MSD, Agilent Technologies). Inlet temperature was set at 280 °C and pressure 14.9 kPa. Sampling volume for analysis was 1 µl in splitless mode with a carrier gas (He) flow set at 2 ml/min. Separation was performed on a DB-1 capillary column 30 m × 0.53 mm × 0.1 µm. The initial separation temperature was 120 °C and increased with a gradient 15 °C/min to 350 °C and held at this temperature for 1 minute. The analyzes were run in SIM mode with selected *m/z* ions: 308, 331, 343, 361, 414, 471, 502, 559, 575, 647. Solvent delay was 8 minutes. Data acquisition and its evaluation were performed by using MSD ChemStation, Agilent Technologies.

Derivatisation of the samples is an important step for HTGC-MS analysis and was performed in 2 ml vials according to the following procedure. To 50 mg of dried and crushed sample was added 250 µl of acetonitrile, the sample was placed in an ultrasonic bath for 30 minutes. Subsequently, 500 µl of HMDS and 2.5 µl of trifluoroacetic acid were added. The mixture was derivatisated in an open vial using thermo-shaker for 30 minutes at 50 °C. Subsequently 250 µl of BSTFA was added and the sample was derivatisated in a sealed vial for another 30 minutes at 80 °C. The thus-derivatised sample was centrifuged and the supernatant transferred to a clean vial for analysis.

For identification and quantification, a standard solution of mixture of selected flavonol glycoside derivates with a 660 mg kg^{-1} concentration was prepared.

3. Results and discussion

Preparation of the sample, or its derivatisation, plays a significant role in gas chromatography, because it increases thermal stability and increases the volatility of organic compounds containing active hydrogen. Silanisation was performed by the stated procedure such that there is no decomposition of the compounds in the sample, and the physical properties of analytes are suitable for HTGC-MS analysis.

In this work we analyzed various drug forms of four types of nutritional supplements containing cranberry extract, as well as tree kinds of dried cranberries and cranberry jam.

Fig. 2 shows a chromatogram of the separation of the standard solution of flavonol glycosides derivates. From Fig. 2 it is clear that the chromatographic

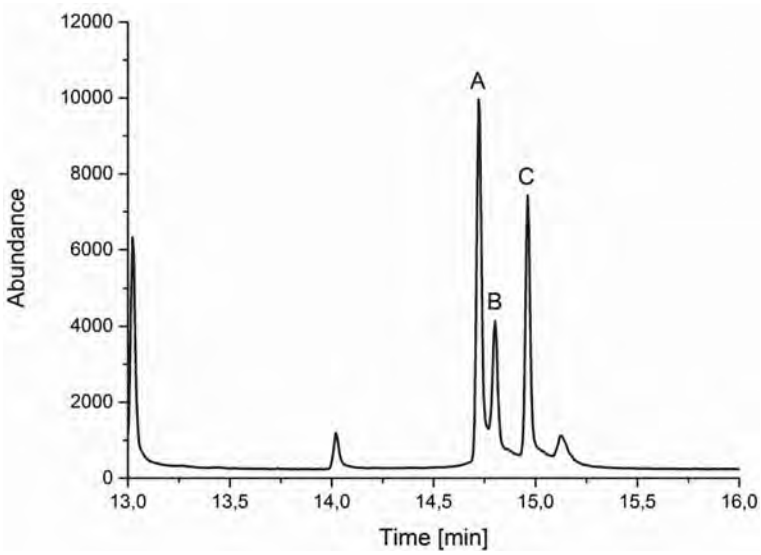

Fig. 2 GC-MS chromatogram of separation of the standard solution of flavonol glycosides: (A) quercetin 3-O-β-D-galactoside, (B) quercetin 3-O-β-D-glucoside, (C) quercetin 3-O-α-D-arabinoside.

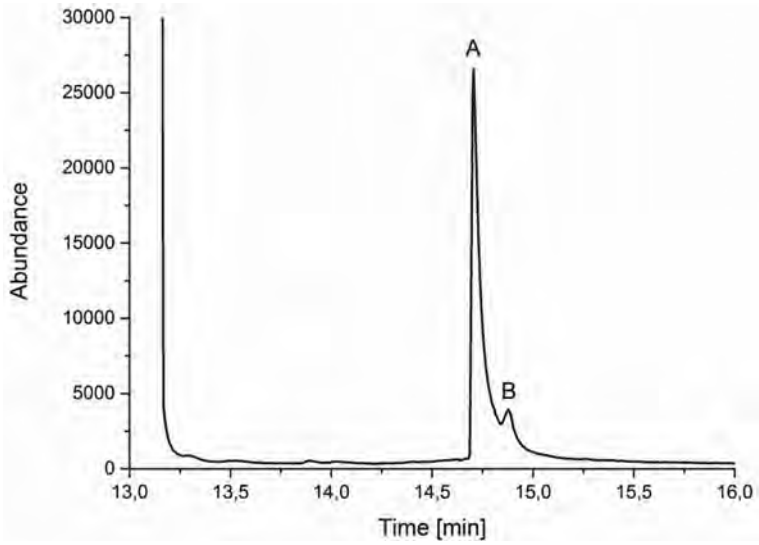

Fig. 3 GC-MS chromatogram from separation of observed flavonol glycosides in sample 3 nutrition supplement: (A) quercetin 3-O-β-D-galactoside, (B) quercetin 3-O-β-D-glucoside.

method we developed for analysis of high-boiling materials is suitable for the separation of flavonol glycosides derivates. The chromatographic system is sufficiently efficient for complete separation, allowing for reliable identification and quantification.

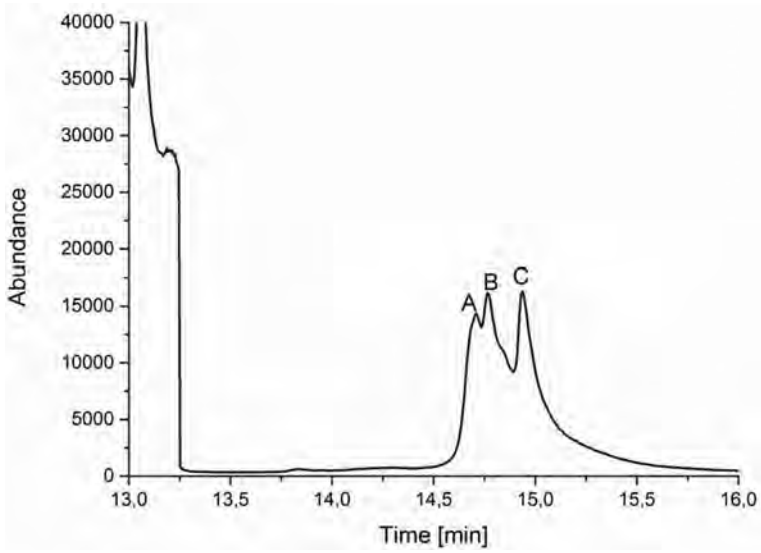

Fig. 4 GC-MS chromatogram of cranberry jam (Cranberry jam): (A) quercetin 3-O-β-D-galactoside, (B) quercetin 3-O-β-D-glucoside, (C) quercetin 3-O-α-D-arabinoside.

In Fig. 3 and 4 are shown the chromatograms achieved from sample 3 nutrition supplement and from cranberry jam. From Fig. 3 it is clear that on the basis of a comparison with the standard in the nutritional supplement, sample 3 contains two flavonol glycoside derivates, a dominant quercetin 3-O-β-D-galactoside and a quercetin 3-O-β-D-glucoside. From Fig. 4 it is clear that all of the flavonol glycoside derivates of quercetin are present in the cranberry jam sample, with a significantly different flavonoid composition as compared to the nutritional supplement. Using these chromatographic conditions however, they were not completely separated. This is due to the presence of other derivates of flavonol glycosides, which is consistent with literature [10, 11]. These substances have MS spectra similar to glycosides of quercetin and therefore in co-elution with glycoside derivates of quercetin they cannot be spectrally differentiated. In Table 1, for this reason, the content of the glycoside derivates of quercetin is stated, together with the co-elutiing substance.

It is evident from Table 1 that the content of the glycoside derivates of quercetin and kaempferol [10] in the measured samples is significantly varied and is in the range of 5–16400 mg kg^{-1}. Due to the low content of the monitored analytes in samples 2 and 3 and their completely different chromatographic profile, these samples can be assumed to be free from *Vaccinium macrocarpon* extract. Although a relatively high content of glycoside derivates of quercetin is measured in sample 3, the chromatographic profile differs significantly from the extracts obtained from dried berries in the Cranberries 1–3 samples. Therefore it can be assumed that this sample does not contain extracts from *Vaccinium macrocarpon.*

Table 1
The total content of the observed flavonol glycoside derivates (quercetin 3-O-β-D-galactoside, quercetin 3-O-β-D-glucoside, quercetin 3-O-α-D-arabinoside) in individual samples.

Sample	Concentration / mg kg^{-1}
Standard solution	660
Sample 1	630
Sample 2	5
Sample 3	1 600
Sample 4	13
Cranberries 1	16 200
Cranberries 2	16 400
Cranberries 3	10 600
Cranberry Jam	4 800

4. Conclusion

A newly-developed analysis procedure for flavonol glycosides has been used to characterize nutrition supplements and the fruit of the American cranberry (*Vaccinium macrocarpon*). This procedure allows the complete separation of these compounds: quercetin 3-O-β-D-galactoside, quercetin 3-O-β-D-glucoside, quercetin 3-O-α-D-arabinoside. We have found that the studied nutritional supplements are significantly different in qualitative composition as well as in quantitative representation. Changes in the content of the glycoside derivates of queretin within a range of four orders suggest that some of nutritional supplements probably do not contain extracts from American cranberry.

Acknowledgments

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Analysis of Candle-Wax by Direct Injection to MS via GC

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Keywords
candles
direct injection
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Abstract
The work presents a new GC-MS analysis of substances with high boiling points, such as candle waxes. Different concentrations of samples were directly injected by into the MS detector the restrictor. Based on the mass fragmentation of characteristic (selective) *m/z* in MS detector, it is possible to distinguish various waxes. The benefit of the newly developed method is short-time analysis with lower temperature and lower values of background compared to the classic GC method.

1. Introduction

Waxes are solid, hydrophobic, organic compounds with melting points over 40°C. Molten, liquid waxes are characterized by the low viscosity used by casting in candle making. Another possibility for producing candles is the use of pressing by high pressure [1, 2]. The most frequently used materials in candles are paraffin, stearin, beeswax, and gel. Beeswax is the product of abdominal bee gallstones consisting of alkyl esters of monocarboxylic acids (72%), free fatty acids (14%), and hydrocarbons (11%) [3]. Stearin consists of palmitic and stearic fatty acids. The paraffin used to make candles is the most common blend of C_{20}–C_{50} alkanes and is a by-product of oil processing. Gel candles are composed of a mixture of mineral oil in the range of C_{20}–C_{50} and a polymeric support: vinyl benzene. Different methods are used to analyze the candle material, e.g., IR, NMR and separation techniques. HPLC-MS analysis of the waxes is characterized by a low yield of analyte [4]. Gas chromatography is the most common method for reliable identification. This method is reliable for identifying materials, but analysis of materials with a high boiling point is limited. The GC-MS connection is limited because some commercial devices do not allow the use of temperatures higher than 350 °C. For this reason, EI ionization is the most often used. MS detection with direct sample input is appropriate for a full analysis of high boiling materials.

However, such a device does not allow the use of an autosampler and therefore its spread in laboratories is very limited.

Gas chromatographic devices allow flexibility of configuration and lead to the development of new methods of analysis. Direct injection analyzes are primarily associated with liquid chromatography, but there also exist methods where the sample is directly injected into the GC system [5–7]. Gas chromatography mainly serves to separate the components of the mixture. In this work, however, gas chromatography is used as a means for direct injection of the sample into the MS without prior separation to GC.

The aim of this work is to develop a new method of analyzing candle waxes using a commercial GC-MS. The newly developed method allows the analysis of high boiling substances that cannot be analyzed by commercial GC-MS. This newly developed method will greatly reduce analysis time and precisely determine the material of candle waxes. The method is based on replacing the chromatographic column with a silanized restrictor. The restrictor will enable the sample to be transferred from the injector to the MS detector.

2. Experimental

2.1 Reagents and chemicals

Chloroform and 2-chloronaphthalene were bought from the J.T. Baker (The Netherland). Stearic, gel, paraffin, and beeswax candles were bought on the local market. The wax samples are dissolved in chloroform with 0.1% 2-chloronaphthalen. All samples were prepared in concentrations 10.00, 5.00, 1.00, 0.50, 0.25, 0.10, 0.05, and 0.01% (*w/w*).

2.2 Instrumentation

GC-MS analysis was performed using a Trace GC Ultra gas chromatograph with a TriPlus autosampler and a TSQ Quantum XLS mass spectrometer (Thermo Fisher, USA). The injector temperature was 350 °C; the MS-transfer line was 350 °C. The compounds were injected through restrictor with length 2 m and internal diameter 50 μm, Agilent Technologies (CA, USA) at a constant flow of 1.1 ml min^{-1} of helium as the carrier gas. Three μl of samples were injected into a splitless mode injector. The restrictor temperature was set at 350 °C. The main parameters related to mass spectrometer settings were ion source temperature 230 °C, electron energy 70 eV, and emission current 50 μA.

GC-FID analysis was performed by gas chromatography, Agilent Technologies 6809N Network GC System with a flame ionization detector Agilent Technologies (USA). Separation of the substances took place on the column UNIMETAL 8.5 m × 0.53 μm i.d × 17 μm, Chromosorb. One μl sample was injected by split mode 5:1 at temperature of injector 380 °C and pressure of 7.3 kPa. Separation

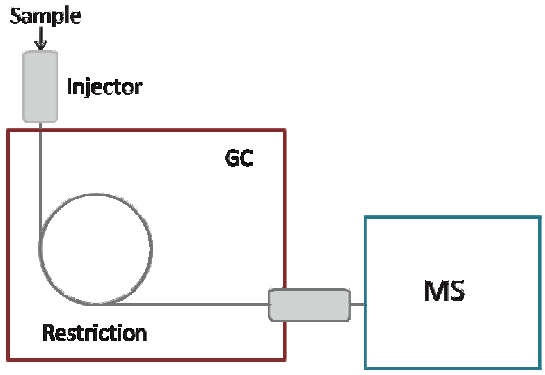

Fig. 1 Schematic diagram of GC-MS system without chromatographic separation.

temperature ranged from 80 °C to 380 °C, with a temperature gradient 10 °C min^{-1} with constant flow.

3. Results and discussion

In Fig. 1 is a schematic diagram of newly developed equipment for the analysis of materials with a high boiling point. The device consists of a gas chromatograph with a restrictor and a mass spectrometer. A 50 µm and 2 m long cutter allows the sample to be transferred from the injector to the detector without chromatographic separation. The interface allows the carrier gas to flow to the detector at 0.01 ml min^{-1} at a pressure of 30 kPa up to a flow rate of 0.07 ml min^{-1} at a pressure of 650 kPa. Compared to the classical chromatographic column, the achieved carrier gas flow values are 10–100 times lower, so the analysis takes place in the high vacuum area of the MS system. Reduced carrier gas flow ensures that the ion source and detector are not overloaded. At the same time, we thus prevent the burning of the filament during sample dosing without the use of solvent delay.

In Fig. 2 (next page) is shown the chromatographic record of an internal standard analysis. The volume of sample stream generated in the injector at 3 µl is less than the liner volume. In splitless mode, it is necessary to wait until the equilibrium concentration in the mixture of the sample and the carrier gas is equal throughout the entire liner volume. This time depends on the volume of the sample, the temperature and pressure in the injector, the type of solvent used and the boiling point of the individual components of the sample. In the case of candle wax analysis, the qualitative composition of the eluate is not changed at 1 minute after dosing. Fig. 2 shows 1 to 2 minute section, which has been used for quantitative and qualitative evaluation of the records.

From the decrease of the response from the record in Fig. 2 it is clear that the concentration of the analyte in the injector varies as a result of the flow of the mobile phase into the MS detector and diffusion into the other parts of the injector.

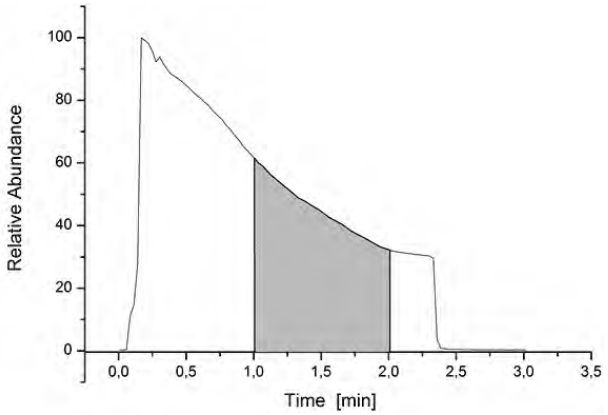

Fig. 2 Chromatographic record after dosing with 3 μl internal strandard with marked area of sample integrals.

We use the GC-FID classical chromatographic method to characterize the four waxes used, and the records are shown in Fig. 3.

In Fig. 3, it is clear that the classical method makes it possible to determine unequivocally the type of wax used. The disadvantage of this system is a relatively long analysis (30 min) and a low life chromatographic column, due to the high temperature load (380 °C). Paraffin waxes elute in the C_{20}–C_{40} region, beeswax in the C_{25}–C_{60}, stearin in the region of C_{18}–C_{22}, and gels in the region of C_{18}–C_{40}.

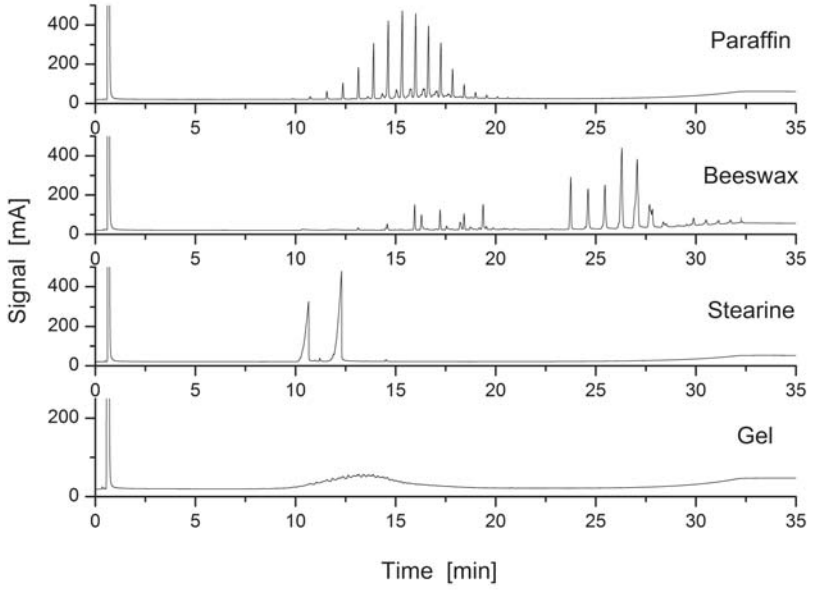

Fig. 3 GC-FID record of wax analysis.

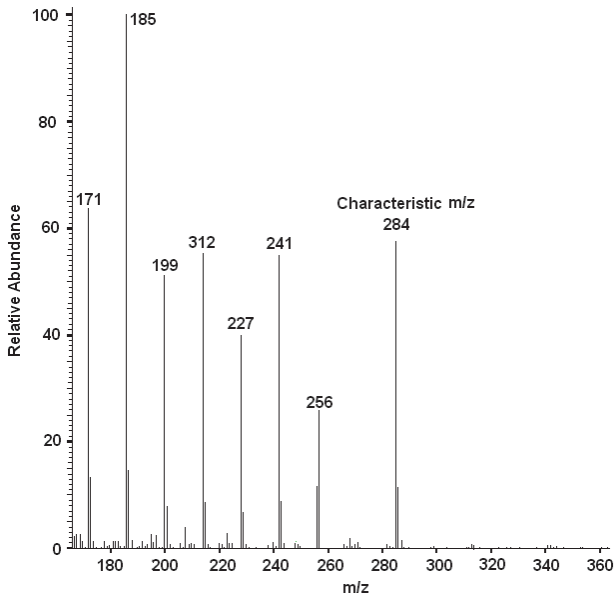

Fig. 4 Selection of the characteristic ion for stearin with mass spectra.

In commercial GC-MS devices, the maximum temperature of transfer line is often 350 °C, therefore the use of the method for identifying high boiling substances is limited. The newly developed system does not contain a stationary phase and thus does not stabilize the balance between the sample and the stationary phase, which allows the migration temperature of the analytes to be reduced. The absence of a stationary phase allows the direct injection of the sample to the MS detector via a restrictor. The determination of the sample takes place only in the MS detector by using characteristic *m/z*.

In Fig. 4 is illustrated the mass spectrum of the stearin solution in chloroform. For characterization of stearin, the characteristic mass of the *m/z* = 284 fragment was selected from the mass spectra. The fragment corresponds to the weight of the stearic acid with the empirical composition $C_{17}H_{35}COOH$. In the same way, we determined *m/z* for other types of waxes. The weight profile obtained provided information on the qualitative composition of the sample. For qualitative purposes, we compared the intensity ratios of selected ions 284, 318, 323 and 593 in the sample. The possibilities of using the newly developed analyte quantification system were characterized by the response of the selected fragment in the individual samples, in dependence on the sample concentration.

In Table 1 (next page) are listed the statistical parameters for different sample concentrations. *RSD* values are given for those samples with a concentration of 1% wax. It is clear from the table that the calibration dependencies for individual materials are linear in the 2-order range, as evidenced by the determination coefficient R^2, whose values are in the range of 0.998 to 0.999. *LOD* and *LOQ* depend on the ion used and the characteristic mass spectrum for individual

Table 1
Characteristic *m/z* for each type of sample with values of line (*a*), shift (*b*) and R^2 from the calibration curve of waxes, and calculated limit of detection (*LOD*) and relative standard deviation (*RSD*).

Compound	*m/z*	$a \times 10^3$ / count s mg ml^{-1}	$b \times 10^3$ / count s	R^2	*LOD* / mg ml^{-1}	*RSD* / %
Stearin	284	26200	–3270	0.998	0.1	3.5
Paraffin	323	4820	–291	0.999	0.06	3.6
Beeswax	593	3240	–45	0.999	0.02	5.7
Gel	318	147	50.6	0.999	0.4	5.5

samples, so its values are markedly different for the individual waxes. The repeatability of analyzes expressed by *RSD* ranges from 3.5 to 5.7. The *RSD* values thus measured indicate that the newly developed system is characterized by a high repeatability of individual measurements.

4. Conclusions

The newly developed system for measuring high boiling point samples in the GC-MS system without the use of a column as a separation unit can be applied to all types of samples analyzed by the GC method. The advantages of the system are a shorter period of analysis (3 min versus 30 min) and the possibility to measure high boiling substances that are difficult to measure by GC-MS. The mass spectra obtained are not loaded with background from the stagnant stationary phase. However, a disadvantage of the system is the fact that a mixed mass spectrum of the whole analyzed sample is obtained, which in some cases may be limiting. We have used the system to identify the materials used to produce the candles, achieving the same quality outputs as when using high-temperature gas chromatography with FID detection.

Acknowledgments

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Analysis of Triglycerides in Detecting Butter Fraud by Low Pressure GC-MS

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Keywords
adulterated butter
LPGC-MS
triglycerides

Abstract
The newly developed method allows the analysis of butter adulterated by sunflower oil using low-pressure gas chromatography - mass spectrometry (LPGC-MS). The method is based on the quantification of individual triglycerides in the samples by mass spectrometry after separation by low pressure gas chromatography, where the compounds elute at lower temperatures compared to conventional gas chromatography. This method allows sunflower oil detection / identification already at 0.5% level of addition in butter.

1. Introduction

Fat from plant and animal product contains different amounts of various fatty acids [1]. At present, despite the control of all types of food, food adulteration is very common, especially in higher quality foods such as milk and its products. For this reason it is necessary to develop a method suitable for detecting cheaper vegetable and animal fats in milk.

One of the first official EU analytical methods for recognition of foreign fats in food was the Precht method. The Precht method was based on the separation of triglycerides in the GC column. This method, due to the high number of various triglycerides isomers, is not capable of separating individual triglycerides from each other; the eluting peaks are, therefore, composed of mixtures of triglycerides with similar molecular weights [2, 3]. In recent years, several alternative methods have been developed on the official but outdated Precht method, using short non--polar capillary columns, which gives the same results as the original packed columns. For analysis of oils and fats, reverse phase high performance liquid chromatography with evaporative light scattering detector was also developed, which is suitable for triglycerides separation [4].

Although the use of liquid chromatography allows the analysis of triglycerides with fatty acids higher than C_{18}, the low separation efficiency limits the use of this method, especially for analysis of triglycerides in butter. To solve this problem, it is appropriate to use gas chromatography. One option is the Low Pressure Gas Chromatography (LPGC) method, which has been successfully optimized for analyses of pollutants in the food industry as well as in the environment, for example: analysis of polycyclic aromatic hydrocarbons, hydrocarbons and volatile organic compounds, volatile aromas and industrial pollutants [5].

A typical LPGC arrangement involves using a short micro-bore column on the injector side connected by a very low dead-volume connector to a magabore column, to be used with higher gas velocities. In such arrangement, injection of the sample at atmospheric pressure is ensured, while the sample separation runs under reduced pressure [6]. Viscosity of the carrier gas decreases with decreasing pressure, which means a shift of the optimal linear velocity to higher values [5]. Higher flow rate reduces retention time and therefore shortens the analysis. There is also a decrease in temperature when the substance elutes from the column. The LPGC system reduces chromatographic peak diffusion to increase the signal/noise ratio (which leads to lower detection limits if there is low matrix effect). In the great amount of work devoted to low-pressure GC, this method has not yet been used for the possibility of high-boiling analysis, although it allows the elution temperature to be about 40 °C, much lower than with conventional gas chromatography.

The aim of this work is to develop a new analytical method with the possibility of monitoring butter adulteration with vegetable oil using low pressure gas chromatography.

2. Experimental

2.1 Reagents and chemicals

Chloroform was purchased from the J.T. Bakter company (The Nederland). Butter and sunflower oil were bought on the local market.

2.2 Instrumentation

LPGC-MS analysis was performed using gas chromatograph Trace GC Ultra with a TriPlus autosampler and a TSQ Quantum XLS mass spectrometer (ThermoFisher, USA). Inlet temperature was 340 °C; the MS-transferline was at 350 °C. Compounds were passed through a silanizated restrictor (20 cm×0.53 mm) at constant pressure 200 kPa of helium as the carrier gas. Two µl of the sample were injected into an injector working in split mode (1:20). The thermal program was started at 80 °C (hold 1 min) and terminated at 370 °C (hold 4 min), with a temperature gradient of 20 °C min^{-1}. The main parameters related to the mass spectrometer setting were: temperature of ion source 230 °C, electron energy 70 eV, emission current 50 µA.

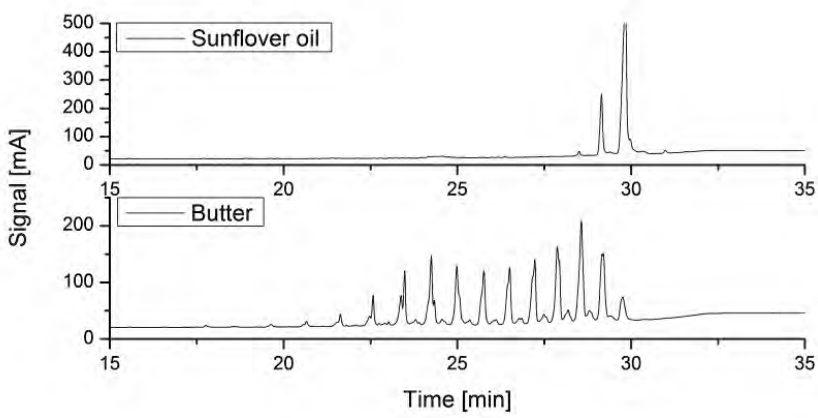

Fig. 1 Chromatogram of butter and sunflower oil using GC-FID method.

GC-FID analysis was performed on a gas chromatograph, Agilent Technologies 6809N Network GC System with a flame ionization detector Agilent Technologies (USA). Computer equipment with program to data acquisition ChemStation rev. B. 04.03 Agilent Technologies (USA). Separation of compounds took place in a column UNIMETAL 8.5 m × 0.53 μm i.d × 17 μm Chromosorb. Sampling volume was 1 μl, injector temperature was 350 °C, and pressure in the injector was 7.3 kPa. Separation proceeded from 80 °C to 380 °C with a temperature gradient of 10 °C min^{-1} at constant flow.

3. Results and discussion

A classic high temperature gas chromatography with FID detector and a low pressure gas chromatography with MS detector were used to analyze triglycerides from butter and sunflower oil. From Fig. 1 it is clear that triglycerides in sunflower oil contain two main groups. We can see from the ratio of individual fatty acids in sunflower oil that the first group contains three C_{18} fatty acids, while the second group contains two C_{18} fatty acids and one C_{16} fatty acid. However, each group contains a large number of different triglycerides, because C_{18} fatty acids are mainly linoleic, oleic and stearic acid. From the chromatogram of butter it is evident that it contains large amount of various triglyceride isomers produced by the combination of C_4–C_{18} fatty acids. Because the FID detector does not allow a distinction between the unsaturated and saturated acids in triglycerides, we used MS detection for their separation. Direct use of MS detection in classic GC for this type of high-boiling substances is difficult, because it allows a transfer line temperature up to 350 °C. During the separation of high boiling-point compounds at a temperature higher than the transfer temperature, the result is sample condensation. The consequence is deficit of analytes and distortion of the results.

The solution is to use LPGC, which is able to decrease the boiling point of these compounds and shorten the time of analysis. A schematic diagram of the

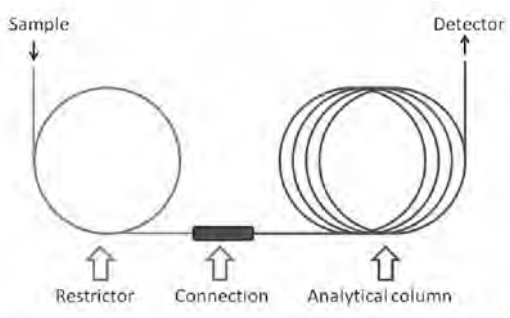

Fig. 2 Scheme of low pressure gas chromatography (LPGC).

connection of the LPGC device used is in Fig. 2. The device is formed of a short restrictor with a silanized surface for minimal adsorption in the stationary phase. The length and diameter of the restrictor is chosen so as to provide a megabre flow rate of 1 ml min^{-1} with the use of standard pressures in the injector. The flow rate and the column's connection to the vacuum in the MS detector ion source result in a reduction in pressure and an increase in the flow rate of the carrier gas in the column. The effect is to shorten the elution time of the triglycerides. The advantage of the chosen system is that the part of the column present in the transfer line is in a high vacuum and therefore does not condense the analytes, even at lower transfer line temperatures than in the chromatographic oven.

Fig. 3 depicts the LPGC-MS chromatogram of triglycerides. The MS record (SIM mode) corresponds to the fragment of triglycerides with two unsaturated fatty

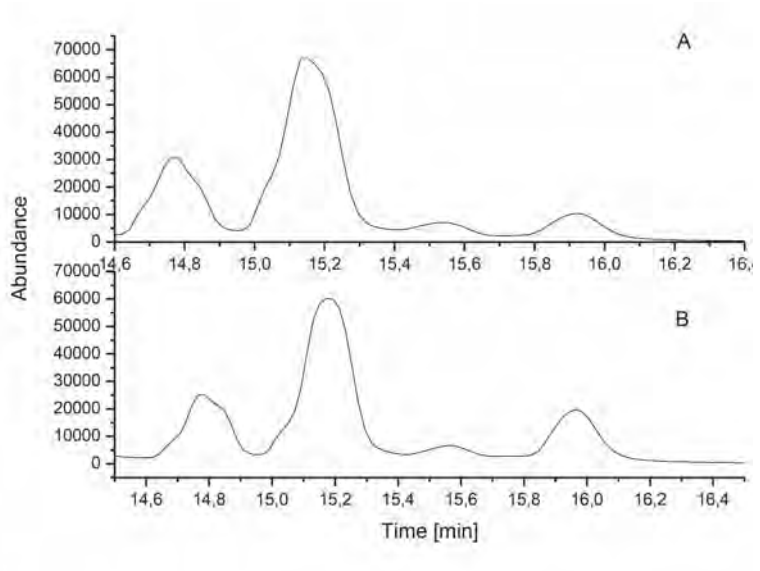

Fig. 3 GC-MS chromatogram (A) 1% solution of butter, (B) 1% solution of butter with 5% solution of sunflower oil.

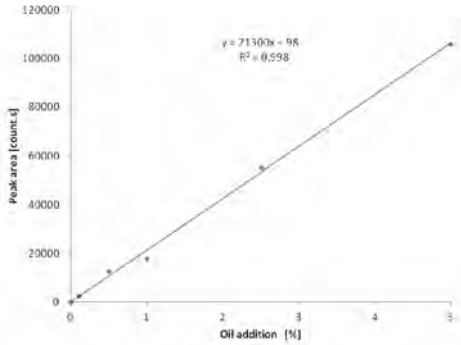

Fig. 4 Calibration curve of the addition of sunflower oil to butter with a range of concentration from 0.1% to 5% by LPGC.

acids with a number of C_{18} carbons. From a comparison of the chromatograms from Fig. 3 it is clear that the addition of vegetable oil to butter significantly increased the peak in at which the triglycerides with three C18 acids are eluated. It is clear from the separation of the individual triglycerides that low pressure gas chromatography can completely separate the individual triglycerides, which differ in the number of carbons atom in the molecule. From Fig. 4 we can see that with the addition of vegetable oil the area of peaks increase linearly and the result is high $R^2 = 0.998$. With this method it is possible to clearly determine the amount of sunflower oil in the butter at a level of 0.5%.

4. Conclusions

This newly-developed method for monitoring the adulteration of butter by sunflower oil is capable of detecting amounts from the 0,5% level of added oil. The method is based on using LPGC with MS detection. With the use of this method, the decrease of the elution temperature of triglycerides and detection of triacylglycerides by MS detector were successfully achieved.

Acknowledgments

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Development of a New Procedure of Gold Electrode Preparation for Electrochemical Application

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Keywords
cyclic voltammetry
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gold cleaning
self-assembled monolayers

Abstract
This work presents the results of optimization of a cleaning method of gold electrodes used in SAMs-based electrochemical measurements. Six cleaning procedures based on the use of polishing pads and diamond suspensions of various grain sizes (3.0, 1.0, 0.5 and 0.1 μm) were tested. The influence of electrochemical cleaning based on cyclic voltammetry measurements performed in sulphuric acid and potassium hydroxide solutions on metal surface purity were investigated. The efficiency of the proposed pretreatments was evaluated by determination of roughness factor. The research has shown that the use of potassium hydroxide solution in cleaning procedure allows to obtain a satisfactorily clean gold electrode surface with good reproducibility and minimizes the number of SAMs defects. Electrochemical measurements using the SAMs-based capacitance sensor demonstrate that the method of cleaning the electrodes has a significant effect on the sensitivity and reproducibility of the obtained results.

1. Introduction

In recent years, a large increase in application of sensors based on Self-Assembled Monolayers (SAMs) in chemical analysis has been observed. Due to highly active surface, which can be easily modified for example by self-adsorption of thiol molecules, gold is one of the most commonly used metal in development of SAMs-based electrochemical sensors. The significant limitation of this material is high risk of impurities adsorption in case of gold exposure on external factors. Contamination of the metal surface affects the binding kinetics of thiol molecules on gold and interfere monolayer adsorption process, as well as its properties. Therefore, it is necessary to clean the metal surface every time before chemical modifications to ensure the formation of the most reproducible monolayers [1, 2].

The surface purity can be verified by cyclic voltammetry measurements (CV), in which such compounds as potassium chloride, potassium hexacyanoferrate or sulfuric acid are used as electrolyte solution [3]. Based on voltammagrams

recorded in sulfuric acid solution, it is possible to calculate roughness factor, which can be defined as the ratio of the real metal surface to its geometric surface. Real electrochemical metal surface can be determined as the area of gold oxides reduction peak divided by potential scan rate and density of electrical charge (for gold 482 μC cm^{-2}). The surface is considered to be satisfactorily clean when roughness factor is in the range between 1.0 to 1.5. Greater value suggests occurrence of surface defects like scratches or increased surface irregularities. If roughness factor is lower than 1.0 it is usually related to presence of impurities on gold surface [4].

2. Experimental

2.1 Reagents and chemicals

The following reagents were used: diamond suspension with grain size 3, 1, 0.5 μm (Leco, USA); diamond suspension with grain size 0.1 μm (Struers, United Kingdom); sulfuric acid 95–97% (Merck, Germany), potassium hydroxide 99% (Centro-chem, Poland); potassium chloride 99.5% (Poch, Poland); sodium nitrate 99.5% (Merck, Germany); nitric acid 65% (Merck, Germany); 18-crown-6 thiol ether, synthesized at the Department of Organic Chemistry, Faculty of Chemistry, Jagiellonian University, Poland; distilled water derived from an HLP 5 system (Hydrolab, Poland).

2.2 Instrumentation

In conducted research CMTR-243 meter (KSP Elektronika Laboratoryjna, Poland) was used. The measurements were carried in three-electrode system in quartz vessel covered with a plastic cover with four holes that matched the size of each electrode and an additional hole for adding standard solution. Gold electrode consisting of 3 mm gold wire, placed in Teflon holder (MTM Anko M10X1) played a role of working electrode, silver chloride electrode with double coat (Ag/AgCl, saturated KCl/2 mol L^{-1} $NaNO_3$; MTM Anko M6) was used as a reference electrode and platinum plate placed in Teflon holder was used as an auxiliary electrode. Also ultrasonic bath Sonic-3 (Polsonic, Poland), miniature magnetic stirrer (Wroclaw University of Technology, Poland) and polishing pads (Leco, USA) were used.

3. Results and discussion

The starting procedure of cleaning gold surface was based on a combination of mechanical and electrochemical stages. At first, the electrode surface was polished manually, by making octal moves on polishing pad soaked with diamond suspension with a grain size of 0.1 μm for four minutes. After that the electrode was carefully washed with water and electrochemically cleaned using a cyclic

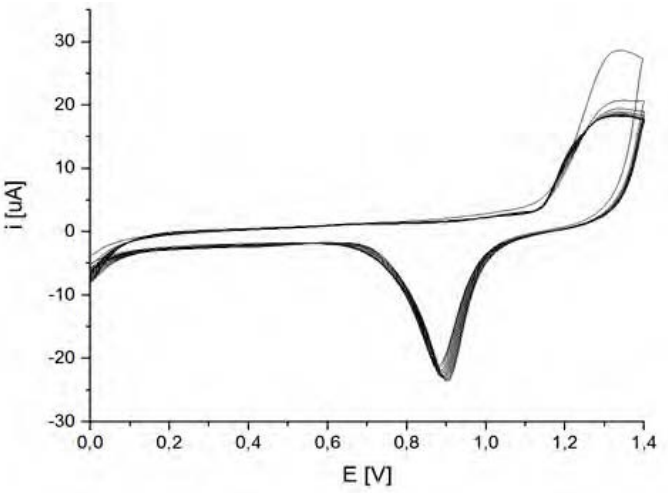

Fig. 1 Cyclic voltammagram obtained during electrochemical cleaning of gold electrodes in sulfuric acid solution (scan rate 100 mV s^{-1}).

Table 1
Applied modifications of the gold electrode cleaning procedure.

Procedure	Mechanical cleaning	Electrochemical cleaning	SAMs adsorption
I	Polishing on pad 0.1 μm	CV in H_2SO_4	–
II	Polishin on pads 0.5 μm and 0.1 μm	CV in H_2SO_4	–
III	Polishing on pads 3.0 μm, 1.0 μm, 0.5 μm and 0.1 μm	CV in H_2SO_4	–
IV	Polishing on pads 0.5 μm and 0.1μm	CV in H_2SO_4	+
V	Sonication in KOH solution, polishing on pads 0.5 μm and 0.1μm	CV in KOH, CV in H_2SO_4	+
VI	none	CV in KOH, CV in H_2SO_4	+

voltammetry technique. Electrodes were subjected to twenty potential cycles from 0–1400 mV at 100 mV s^{-1} scan rate with 4 mV potential step in 0.5 mol L^{-1} solution of sulfuric acid. Exemplary voltammagrams are shown at Fig. 1. Based on the obtained cathode peak area, associated with reduction of gold oxides, roughness factor (R_f) was calculated. Then number of polishing pads were increased according to procedures described in Table 1 (Procedures I – III). Each procedure was repeated ten times and then the average value of R_f was calculated with the confidence interval with 95% level of significance. The final results are presented on Fig. 2. Conducted studies shown that using only one diamond suspension for mechanical cleaning does not provide adequate purity level. As can be seen from Fig. 2, average roughness factor for procedure I is significantly lower than 1.0 what proves presence of surface contaminations. Average R_f values for procedure II and

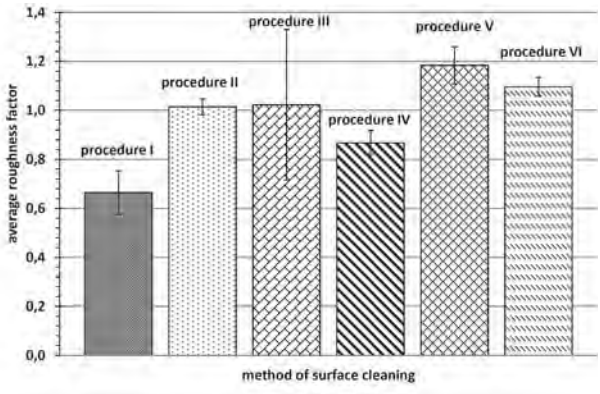

Fig. 2 Purity of electrodes surface according to used procedures of cleaning (from Table 1).

III are very similar and they indicate a satisfactory purity of gold surface. However, the confidence intervals show that using two polishing pads and diamond suspensions with grain size 0.5 and 0.1 μm provides more reproducible surface, while procedure based on using four polishing pads characterizes much worse reproducibility.

After choosing the best procedure of mechanical cleaning the influence of thiol monolayer deposition on the electrode surface was investigated. For this purpose, the purified electrode was immersed for 30 minutes in 0.5 mmol L^{-1} solution of 18-crown-6 crown ether. Subsequently, the electrode was again cleaned by using procedure II, what was repeated ten times and labelled as a procedure number IV. Average R_f value obtained for procedure that simulates the process of construction of a SAMs-based sensor is lower than 1.0 suggesting that the application of mechanical cleaning in combination with CV measurements in sulfuric acid solution is not sufficient to remove completely thiol molecules adsorbed on gold surface. Therefore procedure V was tested, which includes additional stage of sonification of the electrode in 0.5 mol L^{-1} solution of potassium hydroxide for 5 minutes and additional CV measurement by performing twenty voltammetric cycles from –200 to +1100 mV at 100 mV s^{-1} scan rate and 4 mV potential step in the same potassium hydroxide solution. Illustrative voltammagrams are shown at Fig. 3 (on next page). This procedure allowed to obtain the highest roughness factor with satisfactory reproducibility. This cleaning method is relatively time-consuming, therefore attempt was made to shorten it. For this purpose mechanical cleaning step has been skipped. Electrodes were cleaned only by performing CV measurements in potassium hydroxide and sulfuric acid solutions (procedure VI). This cleaning method is characterised by an acceptable value of R_f and good reproducibility, therefore procedure V and VI can be used for gold surface preparation.

The developed sensors, prepared on the basis of this both procedures were used in potassium determination using dielectric capacitance measurements [5].

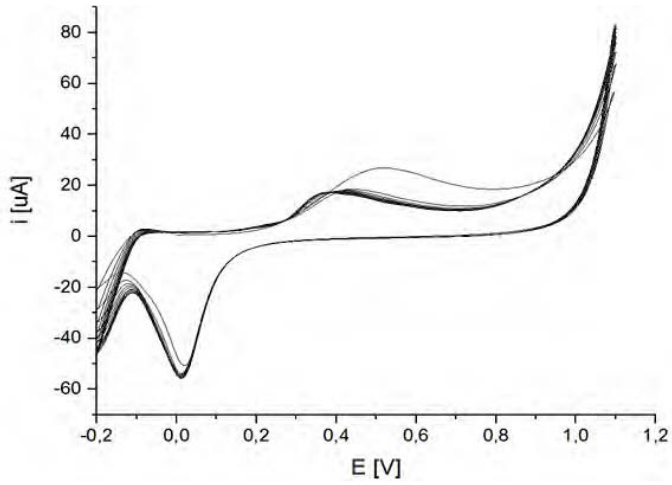

Fig. 3 Cyclic voltammagram obtained during electrochemical cleaning of gold electrodes in potassium hydroxide solution (scan rate 100 mV s^{-1}).

Table 2
Comparison of two procedures of cleaning method obtained for capacitive sensor designated for determination of potassium ions.

Parameter	Procedure V	Procedure VI
Roughness factor	1.187 ± 0.185	1.076 ± 0.028
Sensitivity / nF L $mmol^{-1}$	19.68 ± 4.32	12.86 ± 1.10
RE / %	10.3 ± 1.7	9.3 ± 0.6

The obtained parameters, such as sensor sensitivity and accuracy, expressed by relative error are presented in Table 2. This results show that cleaning procedure containing both mechanical and electrochemical stage guarantees greater sensitivity. It can be observed that way of cleaning gold surface does not affect the accuracy, but application procedure without polishing electrode surface increase reproducibility of sensitivity and precision of obtained results.

4. Conclusions

The conducted study proved that gold surface cleaning method has significant impact on quality of forming thiol monolayers, therefore it also affects sensitivity and precision of applied method. The best results were given by the cleaning procedure comprising sonication in potassium hydroxide solution, polishing on pads using diamond suspensions with grain size 0.5 and 0.1 μm in combination with electrochemical cleaning using cyclic voltammetry in solutions of potassium hydroxide and sulfuric acid. This cleaning method allows to obtain satisfactory purity of gold surface with good reproducibility and also allows to get higher sensitivity, than shorter, electrochemical method of cleaning.

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Pilot Experiments With a Micro-Volume Voltammetric Cell for the Determination of Electrochemically Reducible Organic Compounds

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Keywords
agar membrane
micro-volume voltammetric cell
sodium anthraquinone-2-sulfonate
voltammetry

Abstract
A new micro-volume voltammetric cell for analysis of small volumes of electrochemically reducible organic compounds was developed and tested. The sample (20–100 μL) is placed in a narrow glass tube with an agar membrane at the bottom and the working electrode is immersed into the sample. The agar membrane, as an ion permeable layer, electrically connects the working electrode immersed in the analysed sample in the glass tube with a large-volume compartment (20 mL) filled with Britton-Robinson buffer, where conventional non-miniaturized reference and auxiliary electrodes are placed. The system was tested using a polished silver solid amalgam electrode (p-AgSAE) as a working electrode. Sodium anthraquinone-2-sulfonate was used as a model compound because it is intended to use this system to monitor electrochemically reducible organic compounds.

1. Introduction

The aim of this work was to develop a micro-volume voltammetric cell for analyses of electrochemically reducible organic compounds in micro-volumes of body fluids. Anthraquinone-based anti-cancer drugs belong to the large group of electrochemically reducible organic compounds whose determination in body fluids is highly desirable [1-3]. Their monitoring in body fluids is one of the approaches for their correct dosing and for proper treatment of patients suffering from cancer [4]. Therefore, we have used sodium anthraquinone-2-sulfonate (Fig. 1) as

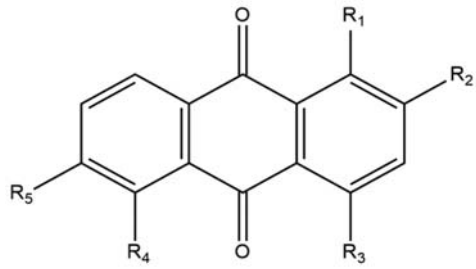

Fig. 1 General structural formula of anthraquinone-derived antitumor drugs (R_x = functional groups or H).

a model substance of anthraquinone-based anti-cancer drugs for testing of the newly proposed micro-volume voltammetric cell (sample volumes from 20 to 100 mL).

2. Experimental

2.1 Micro-volume voltammetric cell

The newly developed electrochemical cell consists of two separated compartments: a micro-volume compartment in which a miniaturized working electrode is placed and a large-volume compartment in which classical non-miniaturized reference and auxiliary electrodes are placed. Both the sample and the working electrode (polished silver solid amalgam electrode (p-AgSAE)) are placed in a narrow glass tube. This tube is immersed into the supporting electrolyte placed in the large-volume (20 mL) compartment containing a reference electrode and an auxiliary electrode. The micro-volume and large-volume compartments are separated by a conductive agar membrane. The thickness and composition of this membrane were optimized and repeatability of its preparation was verified. This cell enables easy work in a three-electrode arrangement.

2.2 Reagents and chemicals

Solution of anthraquinone-2-sulfonate ($c = 1\times10^{-2}$ mol L^{-1}) was prepared by dissolving 0.1551 g of anthraquinone-2-sulfonate (Merck, Germany) in 50 mL of deionized water. Britton-Robinson buffer, as supporting electrolyte for the large--volume compartment as well as for the micro-volume compartment, was prepared by mixing of appropriate volumes of acidic (0.04 mol L^{-1}) and basic (0.2 mol L^{-1}) components. 1 L of the acidic component was prepared from 2.473 g of H_3BO_3 (Sigma-Aldrich, Germany), 2.29 mL of 98% CH_3COOH (Merck, Germany), and 2.5 mL of 85% H_3PO_4 (Merck, Germany); the basic component was prepared by dissolving 8.0 g of NaOH (AppliChem Panreac, Spain) in 1 L of deionized water. 0.1 mol L^{-1} NaCl was prepared by dissolving 0.5844 g of NaCl (Sigma-Aldrich, Germany) in 100 mL of deionized water.

Agar membranes in glass tubes were prepared by dissolving 0.3 g of agar (Fluka BioChemika, Switzerland) in 10 mL of 0.1 mol L^{-1} NaCl and stored in a humidity chamber with 0.1 mol L^{-1} NaCl in a fridge.

All solutions were stored in glass bottles in dark at laboratory temperature, with the exception of the anthraquinone-2-sulfonate solutions which were stored at 5 °C in a fridge. A fresh anthraquinone-2-sulfonate solution was prepared every week.

2.3 Instrumentation

Differential pulse voltammetry (DPV) with following parameters was applied for the determination of anthraquinone-2-sulfonate: polarization rate 25 mV s^{-1}, pulse height –50 mV, pulse width 100 ms, step potential 5 mV, potential range from –350 mV to –900 mV. The determinations were carried out with a PalmSens (Palm Instruments BV, Netherlands) instrument equipped with PSTrace software, version 4.8. This device was controlled by a desktop computer running under Microsoft Windows 10 Enterprise. Measurements were carried out in a three--electrode arrangement with the p-AgSAE (disc diameter of 0.5 mm, lab-made in J. Heyrovský Institute of Physical Chemistry, Czech Republic) as the working electrode, an Ag|AgCl (3 mol L^{-1} KCl) as the reference electrode (Monokrystaly, Czech Republic), and a platinum wire (diameter of 1 mm) (Monokrystaly, Czech Republic) as the auxiliary electrode.

Oxygen was removed by bubbling with nitrogen for 5 min (purity class 4.0, Arliquido, Portugal) before each measurement. The procedure of removing the dissolved oxygen from both large and small compartment was tested, compared, and optimized. All measurements were carried out at laboratory temperature.

3. Results and discussion

DPV at the p-AgSAE was found as a suitable technique for the determination of micromolar concentrations of anthraquinone-2-sulfonate in the constructed micro-volume voltammetric cell. pH = 10 was chosen as optimum (Fig. 2) [5]. Relative standard deviation of 20 repeated measurements of *c*(anthraquinone-2--sulfonate) = 1×10^{-3} mol L^{-1} was about 2%. Under the optimum conditions, the calibration curve was linear in the concentration range from 4×10^{-6} to 1×10^{-3} mo L^{-1}. The obtained equation of the calibration line is

$$I_p[\mu A] = -(1.073 \pm 0.029)\, c\,[\text{mol L}^{-1}] + (-7.8 \pm 11.20) \quad (1)$$

The applicability of the newly constructed micro-volume cell was confirmed by analysis of spiked tap water with addition of Britton-Robinson buffer (final anthraquinone-2-sulfonate concentrations from 10×10^{-6} to 40×10^{-6} umol L^{-1}). Achieved recovery amounted to 95–106%.

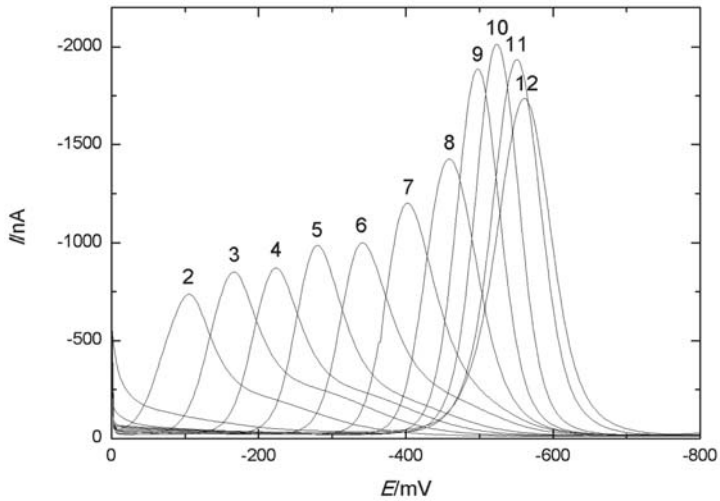

Fig. 2 DP voltammograms of 1×10^{-3} mol L^{-1} anthraquinone-2-sulfonate in Britton-Robinson buffer pH = 2–12 at p-AgSAE; initial potential 0 mV, final potential –1000 mV, scan rate 100 mV s^{-1}, negative regeneration potential –1100 mV, positive regeneration potential +80 mV. Number of regeneration cycles 200; numbers (2–12) above the voltammograms correspond to pH of the Britton-Robinson buffer used [5].

4. Conclusions

A new voltammetric cell for the determination of the model substance (anthraquinone-2-sulfonate) in micro-volumes has been developed. The calibration curve of anthraquinone-2-sulfonate measured in Britton-Robinson buffer (pH = 10) was linear in the concentration range from 4×10^{-6} to 1×10^{-3} mol L^{-1}, with the limit of detection of 7×10^{-6} mol L^{-1}. The constructed micro-volume cell was successfully tested by analysis of 50 µL of model sample of tap water. The achieved results confirmed the practical applicability of the developed cell for sensitive determination of electrochemically reducible organic compounds.

Acknowledgments

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Simultaneous Determination of Antioxidants by Flow-Injection Analysis With Multiple--Pulse Amperometric Detection

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Keywords
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Abstract
A flow injection system and multiple pulse amperometric detection using a glassy carbon electrode were employed to develop and optimize a simple, low-cost, and rapid method for the simultaneous determination of phenolic antioxidants. A sequence of potential pulses was selected in order to detect all targets separately in a single injection step. During the characterization of electrochemical detection, conditions for the determination of antioxidants such as the injected volume and the flow rate were studied, the analytical features such as the repeatability and the calibration characteristics in standard solutions of analytes were measured and determination in real samples was performed.

1. Introduction

Many electrochemical methods, such as cyclic voltammetry [1], differential pulse voltammetry [1, 2], stripping voltammetry [3], and square-wave voltammetry [1, 4] have been used to determine phenolic antioxidants. All these techniques generally have high sensitivity, and are widely used in many areas of analytical chemistry. However, their applicability for the determination of several components in mixtures is limited when the recorded voltammograms display significant partial overlapping.

As a result, techniques preceded by a separation step, particularly HPLC with electrochemical [5], diode-array [6] or MS [7] detection are most frequently used for the determination of mixture of antioxidants. However, application of such complex separation methods might not be necessary in many cases and flow injection analysis (FIA) in combination with a selective detection method might present a suitable alternative. Multiple-pulse amperometry (MPA) has been used

for the simultaneous determination of different analytes [8, 9]. It involves the application of an appropriate potential waveform consisting of a suitable succession of pulses on a single working electrode, thus allowing to distinguish the analytes in a mixture with no need of separation, chemical pretreatment of the sample or electrode modification, or even the use of mathematical techniques for data analysis. This strategy was used for simultaneous determination of sugars [10], drugs [11–13], antioxidants [14], synthetic colorants [15], as well as for the use of internal standard method in FIA [16].

In this work, we demonstrate that MPA detection in combination with FIA system on a GCE electrode can be used for the simultaneous determination of selected antioxidants, namely one pair of sinapic acid and tyrosol, and group of three antioxidants *tert*-butylhydroquinone, propyl gallate, and butylated hydroxyanisole. Results obtained from FIA-MPA method using a flow-cell were evaluated with respect to recovery, repeatability, linearity, and detection limits.

2. Experimental

2.1 Reagents and chemicals

Sinapic acid (CAS Number: 530-59-6), tyrosol (CAS Number: 501-94-0), propyl gallate (CAS Number: 121-79-9), *tert*-butylhydroquinone (CAS Number: 1948-33-0), and butylated hydroxyanisole (CAS Number: 25013-16-5) were supplied by Sigma-Aldrich. Their individual stock solutions (c = 1.00 mmol L^{-1}) were prepared by dissolving the exact amount of the respective substance in methanol (Merck Millipore, Germany) and were kept at 4 °C. More diluted solutions were prepared by exact dilution of the stock solutions with mixture of methanol and 0.040M Britton-Robinson buffer (1:9, *v/v*). All electrochemical measurements were carried out in the same solution. The Britton-Robinson buffer was prepared by mixing 0.20 mol L^{-1} sodium hydroxide (Lach-Ner, Czech Republic) with acidic solution consisting of 0.040 mol L^{-1} boric acid (Lach-Ner, Czech Republic), 0.040 mol L^{-1} phosphoric acid (Merck Millipore, Germany), and 0.040 mol L^{-1} acetic acid (Merck Millipore, Germany). All chemicals used for buffer preparation were of analytical grade purity. Destilled water was provided from a Mega-Pure 3A Liter Automatic Distillation System, USA.

2.2 Instrumentation

All electrochemical recordings were performed using an Autolab PGSTAT12 potentiostat/galvanostat, controlled by NOVA version 1.11.2 software (Metrohm, Switzerland) working under Windows 7 (Microsoft Corporation). The three--electrode wall-jet configuration included a glassy carbon working electrode (GCE; Metrohm, Switzerland, diameter of 2 mm and geometric area 3.1 mm^2), a platinum wire, 1 cm in length and 0.5 mm in diameter, as a counter electrode, and

a Ag/AgCl (3 mol L^{-1} KCl) electrode as a reference electrode (Monokrystaly Turnov, Czech Republic). Flow of the carrier solution was provided by peristaltic pump MINIPULS Evolution (Gilson, USA) and injection of the sample was performed with a six-way injection valve (VICI Valco Instruments, Canada) equipped with a 100 μL sample injection loop. An Orion 266S pH meter (Thermo Fisher Scientific, USA) equipped with a combined glass pH electrode was used for pH measurements. The pH meter was calibrated with aqueous standard buffer solutions at ambient temperature.

Pretreatment of the GCE was done by polishing with alumina powder suspension (0.1 μm) on a damp polishing cloth (Metrohm, Switzerland) before fixing to the flow cell. This procedure was performed at the beginning of the working day. Hydrodynamic voltammograms of sinapic acid and tyrosol were obtained separately by application of nine sequential potential pulses (from +0.40 to +0.80 V for sinapic acid and from +0.70 to +1.10 V for tyrosol, pulse width 100 ms each) in triplicate injections of standard solutions through the FIA system using the MPA technique. The same technique was used for simultaneous amperometric detection of sinapic acid and tyrosol, applying pulses +0.75 V for 100 ms and +1.10 V for 100 ms continuously (total time of the potential waveform was 200 ms).

Hydrodynamic voltammograms of *tert*-butylhydroquinone, propyl gallate, and butylated hydroxyanisole were obtained separately by application of eleven sequential potential pulses (from +0.20 to +0.70 V; pulse width: 100 ms) in triplicate injections of standard solutions through the FIA system using the MPA technique. The same technique was used for simultaneous amperometric detection of *tert*-butylhydroquinone, propyl gallate, and butylated hydroxyanisole, applying pulses of +0.40 V for 100 ms, +0.55 V for 100 ms, and +0.70 V for 100 ms continuously (total time of the potential waveform was 300 ms).

The peak height (I_p) was evaluated from the amperometric FIA recording. The limit of quantification (*LOQ*) was calculated as the analyte concentration corresponding to a tenfold standard deviation of the respective response from ten consecutive determinations at the lowest measurable concentration [17].

3. Results and discussion

In order to identify the potential of oxidation to perform simultaneous determinations of all antioxidants, hydrodynamic voltammograms were first obtained separately for each compound. Also other FIA parameters were optimized; the effect of the injected sample volume on the MPA response, as well as the influence of flow rate were investigated. Under the optimized conditions, repeatability and calibration dependence was measured; ten successive injections of the mixture of a standard solution were carried out. The results demonstrate that the MPA-FIA system provides good repeatability and high throughput (>100 injections h^{-1}). Calibration curves for all targets were constructed using solutions containing

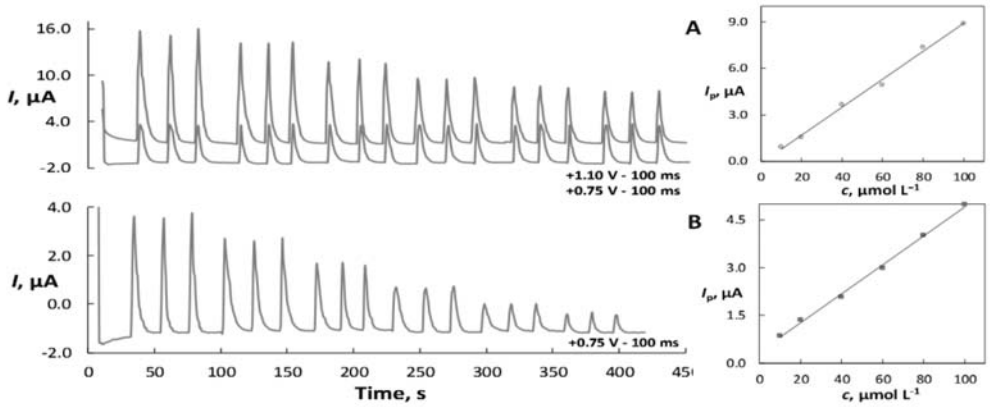

Fig. 1 MPA-FIA recordings obtained after injections of
(A) tyrosol, six standard solutions (100–10 µmol L^{-1}) + sinapic acid (0.1 mmol L^{-1}),
(B) sinapic acid, six standard solutions (100–10 µmol L^{-1}) + tyrosol (0.1 mmol L^{-1}).
Inset shows calibration curves for tyrosol (•) and sinapic acid (■). Potential pulses: +0.75 and +1.10V for 100 ms each; carrier solution: methanol:0.040M Britton-Robinson buffer pH = 2.0 (1:9, *v/v*); injected volume: 100 µL; flow rate: 3.0 mL min^{-1}.

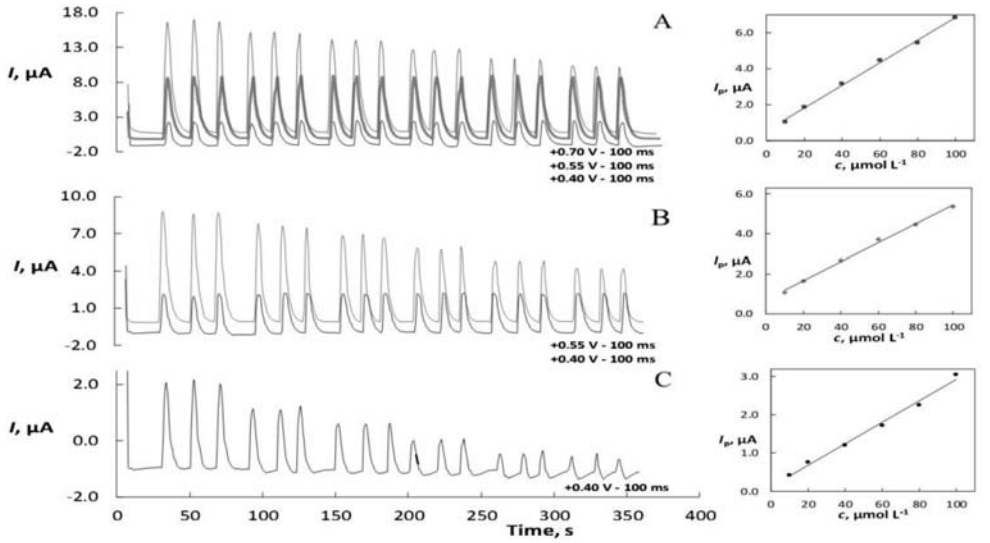

Fig. 2 MPA-FIA recordings obtained after injections of
(A) butylated hydroxyanisole, six standard solutions (100–10 µmol L^{-1}) + *tert*-butylhydroquinone and propyl gallate (both 0.1 mmol L^{-1}),
(B) propyl gallate, six standard solutions (100–10 µmol L^{-1}) + *tert*-butylhydroquinone and butylated hydroxyanisole (both 0.1 mmol L^{-1}),
(C) *tert*-butylhydroquinone, six standard solutions (100–10 µmol L^{-1}) + propyl gallate and butylated hydroxyanisole (both 0.1 mmol L^{-1}).
Calibration curves for *tert*-butylhydroquinone (•), propyl gallate (⧫) or butylated hydroxyanisole (■) are presented on the right hand side. Potential pulse: +0.40, +0.55, and +0.70V for 100 ms each; carrier solution: methanol:0.040 M Britton-Robinson buffer pH = 2.0 (1:9, *v/v*); injected volume: 100 µL; flow rate: 2.0 mL min^{-1}.

Table 1
Figure of merits of the proposed method for the simultaneous MPA-FIA determination of antioxidants (*RSD* is for 10 injections of concentration of 0.1. mmol L^{-1}).

Substance	Concentration range / μmol L^{-1}	Slope / mol^{-1} L	Intercept / μA	*R*	*LOQ* / μmol L^{-1}	*RSD* / %
sinapic acid	0.8–100	47.61	0.211	0.9956	0.86	2.48
tyrosol	1.0–100	89.78	0.076	0.9973	1.03	3.96
tert-butylhydroquinone	2–100	66.59	0.293	0.9955	2.51	0.84
propyl gallate	1–100	51.49	0.408	0.9901	1.45	1.53
butylated hydroxyanisole	0.8–100	28.14	0.114	0.9947	0.85	3.69

varying concentrations of one antioxidant while the concentration of the other remain constant. Figs. 1 and 2 show the amperometric responses of this measurement at one concentration level (10^{-5} μmol L^{-1}) and the calibration plots proving the proportionality between the amperometric current and the concentrations of the analytes. (for more details see Table 1). As an application, the determination of the antioxidants contained in real samples was carried out by applying a simple extraction procedure.

4. Conclusions

The FIA-MPA technique provides short analysis time, low consumption of reagents and samples, high precision and linear calibration curves with limit of detection values at micromolar quantities. Furthermore, the method requires simpler instrumentation and provides lower investment and running cost in comparison with others more expensive techniques, e.g. HPLC with electrochemical detection, diode-array detection, or MS typically applied for simultaneous determinations of more than one antioxidant.

Acknowledgments

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The Numerical Method for Calculation of the Quantitative Composition of Complex Water-Ethanol Solutions

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Keywords
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strength
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Abstract
The numerical method for calculation of the quantitative composition of complex water-ethanol solutions was proposed. It can be used for correct strength determination in any type of alcoholic drink or corresponding precursor. According to the method every complex water-ethanol solution is theoretically divided into hydrous and anhydrous parts. The contraction phenomenon was expressed numerically by the introduction of the function depending on the composition of the solution. The usage of both proposed method and new analytical method "Ethanol as internal standard" is able to improve the quality control of alcoholic beverages.

1. Introduction

An alcoholic drink, or an alcoholic beverage, is a drink that contains a substantial amount of ethanol. There is a great amount of types of alcoholic drinks in the world. The main difference between them lays in the production way and strength values. The strength of an alcoholic drink means the volume of pure ethanol contained in the volume of a drink itself. The determination of the strength of the sample of alcoholic beverage is one of the key actions while routine quality control tests. From the other hand, the determination of volatile compounds contamination is another important test because these impurities can change the taste of alcoholic drink and what is worse some of them are toxic. According to the European commission regulation [1], the algorithm of determination of volatiles contamination lays in gas chromatography way by usage the traditional method of internal standard. Regulation also establishes the presentation of volatiles concentrations in mg/l of absolute alcohol measured units; to transfer obtained results in required units from mg/kg one should determine the strength of the analyzed sample. The fact is that strength determination is proceeded by usage

of international alcoholometric tables [2]. These tables match up the strength of the sample with its density at a certain temperature. It is necessary to mark that alcoholometric tables are suitable for only binary water-ethanol solutions. But what is to be done when there is a necessity to analyze the sample with high contamination of volatiles? Such alcohol drinks as whisky, cognac, brandy, etc. and definitely the distillates from which they are produced contain noticeable amounts of impurities. In these cases it is impossible to determine strength accurately, because direct density measurement is not able to feel the difference between ethanol and for example methanol. This fact creates a problem: how to determine strength correctly?

We have proposed the numerical method for calculation of quantitative composition of complex water-ethanol mixtures, which is able to get around the above-mentioned problem. Firstly we assume that analyzed sample is prepared by mixing two separate solutions. The first solution is pure water. The second solution is an "anhydrous part" of analyzed sample consisting of *n* volatile compounds and ethanol. The density of the sample after mixing its "anhydrous part" with water can be represented by the equation

$$\rho_{\text{mix}} = C_{\text{water}}\,\rho_{\text{water}}^{\text{eff}} + \left(1 - C_{\text{water}}\right)\sum_{i=1}^{n}\rho_i C_i^{*} \tag{1}$$

where ρ_{mix} is the density of the analyzed sample [mg/l], C_{water} is the volumetric concentration of water in the analyzed sample [ml/ml], ρ_i is the density of *i*-th volatile compound or ethanol [mg/l], C_{i}^{*} is the concentration of *i*-th volatile component in anhydrous part [mg/l (absolute alcohol)]. The $\rho_{\text{water}}^{\text{eff}}$ value is called effective water density and appeared to be depending on *F* value

$$\rho_{\text{water}}^{\text{eff}} = \rho_{\text{water}}^{20} F\left(C_{\text{water}}\right) \tag{2}$$

where ρ_{water}^{20} is the density of pure water at 20 °C [998230 mg/l] and *F* is the function depending on the volumetric concentration of water in the sample. Actually *F* is the numerical expression of a contraction phenomenon. The fact is that while mixing pure components (ethanol and water) the final solution has less volume than the sum of volumes of pure components. The reason is the appearance of intermolecular interactions which lead to the changes of supramolecular structure. This phenomenon is called "contraction". F value can be found by usage of alcoholometric tables [2] as they describe binary water-ethanol solutions and it is possible to receive the following equation by the substitution of Eq. (2) into Eq. (1)

$$F = \frac{\rho_{\text{mix}} - C_{\text{ethanol}}\,\rho_{\text{ethanol}}}{C_{\text{water}}\,\rho_{\text{water}}^{20}} \tag{3}$$

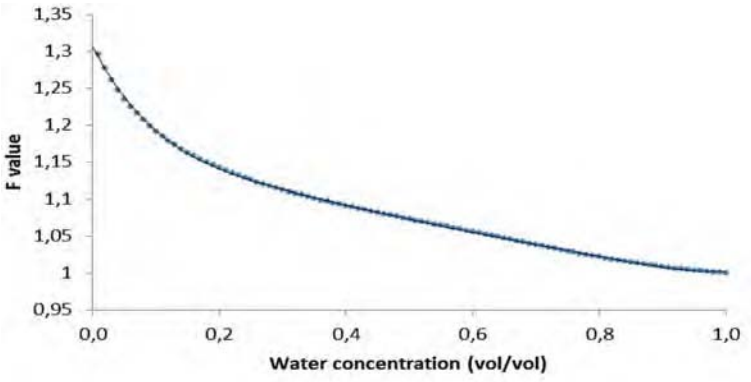

Fig. 1 The dependence of *F* value on the water concentration in water-ethanol mixture.

As it was found, the dependence of *F* value on the concentration of water in the water-ethanol solution is a monotonic function without extrema of the following form

$$F(C_{\text{water}}) = a\,x^6 + b\,x^5 + c\,x^4 + d\,x^3 + e\,x^2 + f\,x + g \tag{4}$$

The graphic form of this function is demonstrated on the Fig. 1.

Values of the volume concentration of *i*-th volatile compound including ethanol in the anhydrous part of the sample in ml/ml may be represented by the following expression

$$C_i^* = \frac{\widetilde{C}_i / \rho_{\text{i}}}{\sum_{i=1}^{n} \widetilde{C}_i / \rho_{\text{i}}} \tag{5}$$

where $\widetilde{C}_i$ is the concentration of *i*-th volatile compound in the anhydrous part of the sample expressed in mg/l (absolute alcohol) which can be determined directly from the chromatographic data by using method "Ethanol as internal standard" [3]. By the substitution of the Eq. (5) into Eq. (1) we can receive

$$C_{\text{water}} = \frac{\rho_{\text{mix}} \sum_{i=1}^{n} \frac{\widetilde{C}_i}{\rho_{\text{i}}} - \sum_{i=1}^{n} \widetilde{C}_i}{\rho_{\text{water}}\, F(C_{\text{water}}) \sum_{i=1}^{n} \frac{\widetilde{C}_i}{\rho_{\text{i}}} - \sum_{i=1}^{n} \widetilde{C}_i} \tag{6}$$

The volumetric concentration of *i*-th component in the analyzed sample is represented as

$$C_{\text{i}} = (1 - C_{\text{water}})\, C_{\text{i}}^* \tag{7}$$

Name of the sample:

Sample – CRM LGC5100 Whisky-Congeners

Compound	Density*, mg/L	RRF Average**	RRF ☐ Custom	Area, arb. units	Concentration, mg/L (AA)
acetaldehyde	783400	1.337	1.337	31.216	75.844
isobutyraldehyde	793800	1.109	1.109	0	0
ehtylformate	916800	1.321	1.321	0	0
acetone	784500	1.300	1.300	0	0
methyl acetate	934200	1.387	1.387	3.481	8.774
ethyl acetate	900300	1.117	1.117	121.388	246.401
methanol	786600	1.223	1.223	23.757	52.800
2-butanone	805000	0.900	0.900	0	0
2-propanol	785000	0.969	0.969	0.917	1.615
ethanol	**789300**	**1.000**	**1.000**	434338	789300 (39.90 v/v %)
diacetyl	990000	2.019	2.019	0	0
2-butanol	806300	0.853	0.853	0	0
1-propanol	805300	0.679	0.679	471.362	581.619
isobutanol	801800	0.581	0.581	585.582	618.270
isoamyl acetate	876000	0.707	0.707	0	0
1-butanol	809800	0.648	0.648	4.443	5.232
isoamylol	813000	0.632	0.632	775.877	891.095
hexanol	815300	0.600	0.600	0	0
ethyllactate	1032800	1.908	1.908	0	0
cyclohexanol	962400	0.556	0.556	0	0
benzyl alcohol	1041900	0.909	0.909	0	0
phenylethanol	1013000	0.730	0.730	0	0
sample	948060				

Print the results

Fig. 2 The work area of the on-line calculator "AlcoDrinks" (http://inp.bsu.by/calculator/vcalc.html).

The algorithm of calculation lays in the method of successive approximations. Firstly, F value in the Eq. (6) is set equal to 1. Then it is possible to calculate the C_{water} value. The obtained result is zero-order approximation value. Further by using the received amount of water concentration, one clarifies the F value. The actualized F value now differs from 1 and is used again in the Eq. (6). The circle has been closed. Each new iteration clarifies the F and C_{water} values. At any specific time the volumetric concentration of each compound in the analyzed sample can be calculated according Eq. (6). Let us recall, that the C_i^* value can be easily found from GC measurement by using the new method [3].

The algorithm of successive approximations may be successfully done by computer equipment. Thus, we created the Excel document with corresponding calculations. What is more, the on-line calculator „Alco Drinks" was created [4]. It uses the algorithm of calculations described in this article. User must enter the density of the analyzed sample, the GC data (areas of response) and, if necessary, individual relative response factors (*RRF*s). The work area of the calculator is demonstrated in the Fig. 2.

2. Experimental

2.1 Reagents and chemicals

All individual chemical compounds were purchased from Sigma-Aldrich. All the standard solutions were prepared gravimetrically by the addition of individual chemical compounds into water-ethanol solution.

2.2 Instrumentation

The ingredients were separated by GC (Chromatek-5000) equipped with autosampler and flame ionised detector on column 30 m × 0.25 mm × 0.25 μm. The injected volume of the sample was 1 μl. Nitrogen was used as a carrier gas. Injector temperature was set to 190 °C. The oven temperature was set to 70 °C for 9 minutes, gradually increased to 180 °C for 16 minutes and held for 5 minutes.

2.3 Preparation of standard solutions

All water-ethanol solutions were diluted by deionised water. Standard solution "C" for the determination of *RRF* values was prepared gravimetrically in full compliance with [1]. The determination of density was carried out by a pycnometer.

3. Results and discussion

To demonstrate the correctness of the proposed numerical method ten water-ethanol solutions were prepared and corresponding GC measurements were carried out. The strength of the prepared solutions was determined according alcoholometric tables and the proposed method. The results are presented in the Table 1. Obtained results show that proposed method is correct enough in the case of using water–ethanol solutions with very small amounts of volatiles (it is almost impossible to create binary water–ethanol solution).

For the next experimental test we prepared solution which contained water. ethanol and isoamyl alcohol at the volumetric ratio 60:20:20. Then strength was again determined by both traditional and novel methods. The obtained results are 76.3% and 60.5% correspondingly. But the fact is that real volumetric contamination of ethanol is close to 60% and this experiment shows the limitations of classical strength determination. The chromatogram of prepared solution is demonstrated in the Fig. 3. The *RRF* values for the corresponding calculations were found by preparation of standard solution "C" in full compliance with [1] according to the new method "Ethanol as internal standard".

Table 1
Strength determination in binary solutions by both methods.

Method	Strength / %									
Alcoholometric tables	10.0	20.0	30.0	40.0	50.0	60.0	70.0	80.0	90.0	95.5
New method	9.97	19.97	29.96	39.99	49.99	59.99	69.99	79.99	89.95	95.49
Deviation / %	–0.03	–0.03	–0.04	–0.01	–0.01	–0.01	–0.01	–0.01	–0.05	–0.01

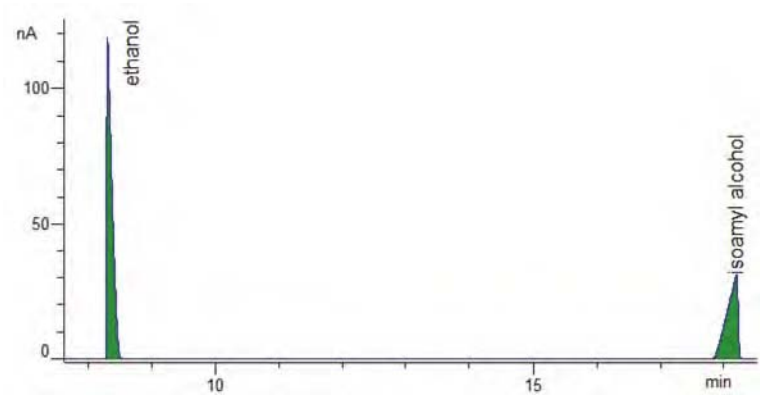

Fig. 3 Chromatogram of the prepared solution with noticeable amount of isoamyl alcohol.

4. Conclusions

The experimental results clearly show the trueness of the proposed method both in cases of binary and complex water–ethanol solutions with different concentrations of volatile compounds. The *RRF* values obtained during analysis of standard solution "C" were calculated according new analytical method. Eventually there is a necessity to combine method "Ethanol as internal standard" with the mentioned here method of calculation of the volumetric concentration of every organic compound in the alcohol beverage. Both new methods have better metrological parameters than traditional. In such a manner we'll receive the unique technique of solving two main tasks during quality control tests of alcoholic beverages: the determination of strength and the determination of volatile compounds contamination.

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Application of Inorganic, Modified and Nanostructured Sorbents for Determination Accuracy Improvement of Biomarkers Quantitative Content in the Exhaled Air

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gas chromatography

Abstract
The aim of this work is the development of calibration methods and techniques for *n*-pentane quantitative determination in exhaled air. The results of the chromato-desorption microsystems development have shown. The systems of interest have been filled with four types of sorbents: inorganic, modified and nanostructured. The developed method can be used for calibration of analytical equipment during clinical testing of risks of health functional diseases.

1. Introduction

Medical technologies for monitoring and diagnosis are the primary tools for differentiating diseases. They have the potential to predict future changes in the human body. Detection of diseases at early stages increases the likelihood of successful therapeutic treatment. This is why the need to create accessibly, quality and rapid ways of the body state diagnosing is a priority in the medical analytical techniques development. Exhaled air is defined as a medium, the analysis of which is a proper direction in non-invasive diagnostics due to the possibility of highly selective and accurate biomarkers determination. These biomarkers are reflecting a broad range of changes occurring in the body [1]. The World Health Organization statistics show that cardiovascular diseases lead to the majority of deaths from noninfectious diseases (17.7 million people per year), which is the reason why a diagnosis of cardiovascular diseases requires special attention [2].

The aim of this work is the development of chromato-desorption microsystems, which are able to perform the calibration of analytical equipment for

n-pentane determination in exhaled air. Due to the fact that a number of studies have shown a correlation between the increase of an *n*-pentane in the exhaled air and the presence of cardiovascular diseases, the *n*-pentane was chosen as the target component [3]. The analytical equipment calibration stage includes one of the most significant errors in the result of the analysis. A chromato-desorption microsystem (ChDmS) proposed in this paper allows making it possible to obtain calibration mixtures with an acceptable level of accuracy.

2. Experimental

2.1 Sorbents

In this study the following sorbents were used: super-crosslinked polystyrene MN–202 (Purolite, UK), Al_2O_3 (Sorbice, RU), carbon nanotubes (Nanouglerodnyie materialyi, RU) and fibreglass (Steklotkan, RU).

2.2 Preparation of modified sorbents

The sorbents MN-202, Al_2O_3, and carbon nanotubes were used in the condition provided by commercial organisations.

The method of applying a stationary liquid phase to a solid carrier was used for creating the fibreglass with a stationary liquid phase 15% PMS-1000. For this purpose, 0.05 g of PMS-1000 stationary liquid phase is dissolved in 48.50 g of boiling methyl benzene, and a solid carrier (0.36 g of glassfibre) is added to the resulting mixture, followed by evaporation of the solvent. In order to apply a stationary liquid phase to solid carriers, the modified fibreglass was placed in containers through which an inert nitrogen gas was carried with the use of the capillaries. The amount of solvent was chosen according to the criterion of the required volume, which should be sufficient to immerse the solid carrier completely. Evaporation was carried out until the solvent was completely removed. Then the sorbent was placed in a laboratory muffle furnace, where it was dried at 150 °C for 3 hours.

2.3 Chromato-desorption system producing and preparation procedure

Production process of chromato-desorption microsystems was carried out in several steps (Fig. 1, next page). At the first stage, the tubular container, which is a medical needle (40 mm in length) with an internal diameter of 0.5 mm, was filled uniformly with sorbents. The second stage is the saturation of the system by the target component. Then, for the distribution of the component through the system, a preparation procedure is conducted. The intensity of the sorbent colour in Fig. 1 corresponds to the saturation degree of the sorbent with the target component.

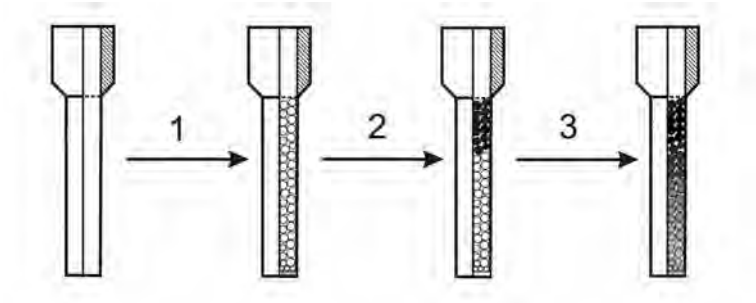

Fig. 1 Stages of chromato-desorption system production: (1) filling with a sorbent, (2) saturation with a target component, (3) preparation procedure.

Creating a concentration gradient and balanced distribution of the analyte in the system can be achieved by conducting a preparation procedure. To implement this procedure, the system is placed in the heating block at a temperature of 120 °C (for 2 min). Then 1.5 ml of clean air is passed through the system evenly for 10 seconds.

2.4 Calibration gas mixtures preparation by a chromato-desorption method

The experiment was carried out on a gas chromatograph Crystal 5000.1 with a flame ionization detector. The separation was carried out on a HP-1 capillary column 30 m long, 0.32 mm in diameter. Temperature of the injection oven was varied in levels 50, 70 or 100 °C for obtaining gas mixtures with different concentrations of target component. Temperature of the column was set on 30 °C and temperature of the detector was hold on 220 °C. The volume of air passing through the system was 0.5 ml. With the purpose of obtaining gas mixtures, the ChDmS was contained into injector oven for 10 seconds. Then 0.5 ml of pure air was carried through the system.

3. Results and discussion

The Fig. 2–4 shows relation between *n*-pentane concentration and the number of consecutively obtained gas mixtures with the use of ChDmS filled with different type of sorbents (excluded a rump-up for a better understanding). Derived relations are corresponded with previously obtained results for standard chromato-desorption systems [4]. Schematic representation is shown on the Fig. 5. Table 1 presents experimental data on the performance of chromato-desorption microsystems.

The studied systems configurations have shown the possibility of obtaining a number of concentrations with a change of the desorption temperature. At the same time, each of the microsystems meets the requirements for calibration gas mixtures for such characteristics as the standard deviation of the obtained mixture concentrations (less than 15%) and the duration of the working plateau (the possibility of maintaining a concentration of at least 3 inputs). At the same

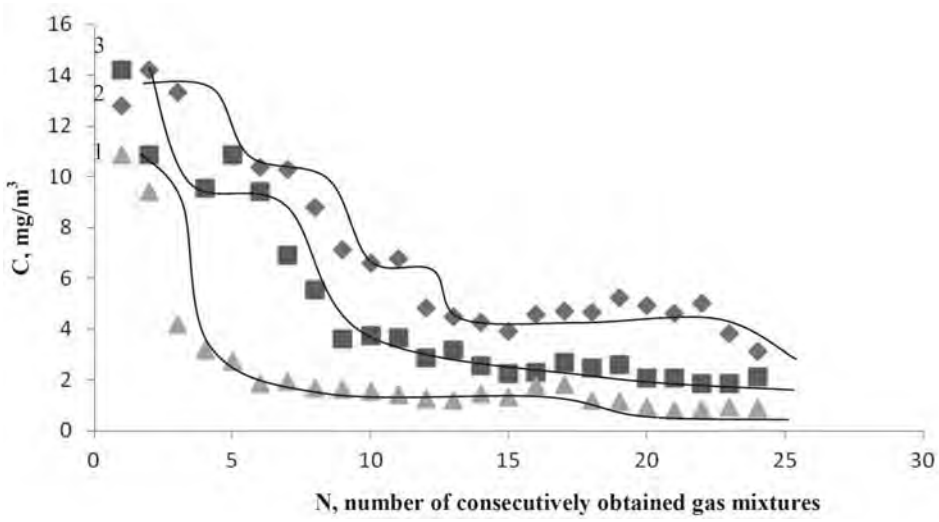

Fig. 2 Relation between *n*-pentane concentration and the number of consecutively obtained gas mixtures with the use of ChDmS filled with fibreglass with 15% PMS-1000: (1) desorption temperature 50 °C, (2) desorption temperature 70 °C, (3) desorption temperature 100 °C.

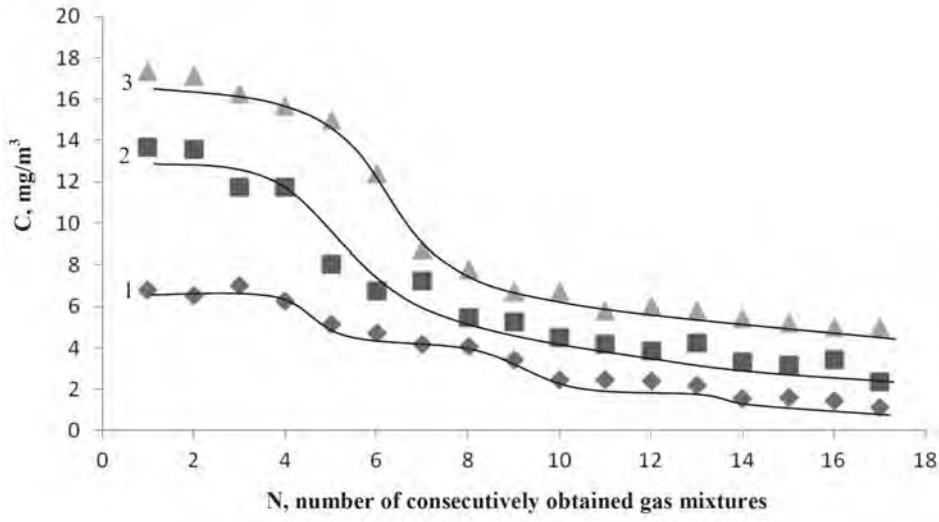

Fig. 3 Relation between *n*-pentane concentration and the number of consecutively obtained gas mixtures with the use of ChDmS filled with carbon nanotubes: (1) desorption temperature 50 °C, (2) desorption temperature 70 °C, (3) desorption temperature 100 °C.

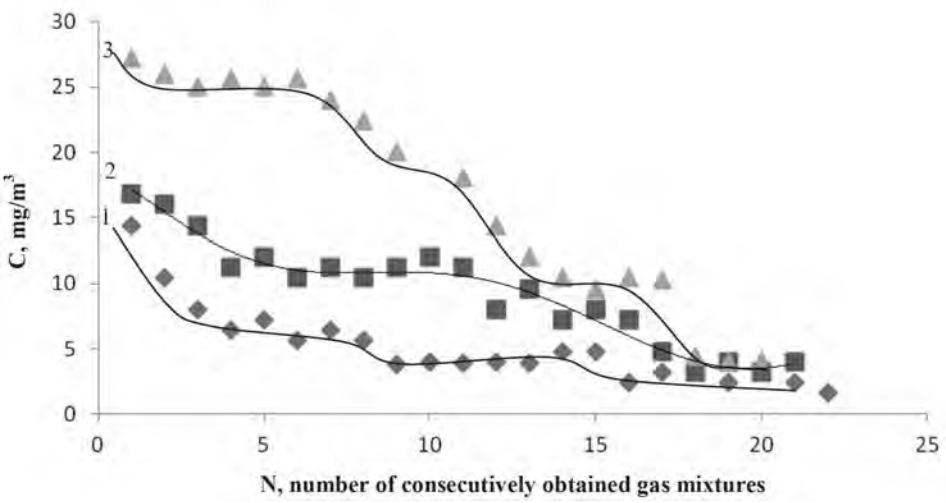

Fig. 4 Relation between *n*-pentane concentration and the number of consecutively obtained gas mixtures with the use of ChDmS filled with Al_2O_3: (1) desorption temperature 50 °C, (2) desorption temperature 70 °C, (3) desorption temperature 100 °C.

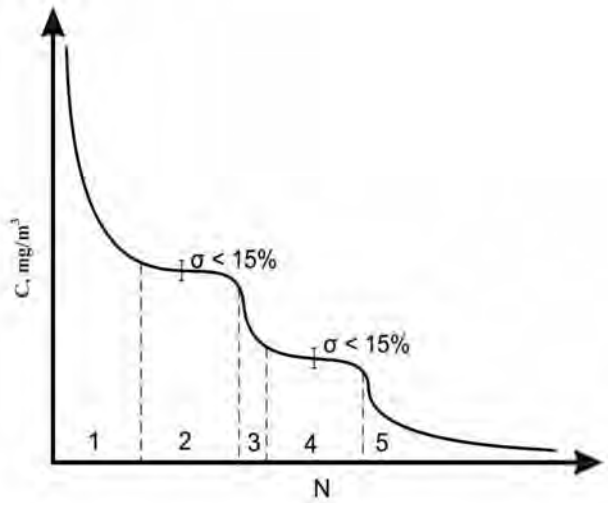

Fig. 5 The relation between the number of consecutively obtained mixtures and their concentration: (1) ramp-up time, (2, 4) constant concentration site, (3) period of redistribution of the target component in the system, (5) system exhaustion period.

Table 1
Chromato-desorption microsystem prototype working results ($t_{des.}$ – analyte desorption temperature, n – number of consecutively obtained mixtures with the constant concentration, C_{mix} – mixture concentration obtained from the system).

Sorbent	$t_{des.}$ / °C	n	C_{mix} / mg m^{-3}	*RSD* / %
Fibreglass with 15% PMS-1000	50	4	2	8
	70	4	10	8
	100	4	14	4
Carbon nanotubes	50	4	7	5
	70	4	12	7
	100	3	17	3
Al_2O_3	50	5	6	10
	70	8	12	5
	100	5	25	2

time, the configurations of systems with carbon nanotubes and Al_2O_3 show the highest stability parameters of the working cycles (average concentrations standard deviation of the resulting mixtures are 5.0 and 5.7%, respectively), and the difference in obtained concentration, which in percentage terms ranges from 40 to 100% of the initial concentration.

4. Conclusions

The chromato-desorption microsystems based on modified, inorganic and nanostructured sorbents have been developed to produce calibration gas mixtures containing n-pentane, by means of which the analytical equipment was calibrated. The system configuration was optimised. The system filled with carbon nanotubes appears the most efficient configuration of ChDmS. The possibility of using microsystems for the calibration gas mixtures preparation has been shown experimentally. The range of gas mixtures obtained was 2–25 mg m^{-3}.

Acknowledgments

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A Study on (Bio)sensor Matrices Based on Titanium Sol-Gel Composites

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Keywords
biosensor
nanocomposite
sensor
titania dioxide sol

Abstract
The main goal of the presented research was to develop a new nanocomposite modifying the surface of graphite electrode. The nanocomposite was based on titania dioxide sol enriched with different components including mesoporous carbon CMK-3, gold nanoparticles and Nafion mixed in different proportions. Based on the cyclic voltammetry measurements the developed sensors with different composites were characterized with respect to sensitivity towards Fe(II)/Fe(III) redox probe. Obtained calibration curves allowed to choose the best nanocomposite modifying the graphite electrode surface that can be employed in development of new (bio)sensors.

1. Introduction

Among the most popular analytical methods the chromatographic and spectroscopic methods should be distinguished. These techniques are used in many pharmaceutical and environmental laboratories in the world and could be employed in qualitative and quantitative analysis. On the other hand application of these methods is related with high cost of apparatus and special sample preparation. The application of electrochemical methods including electrochemical sensors often allows obtaining satisfactory results without special sample preparation or the use of expensive apparatus.

According the IUPAC definition the chemical sensor is a small measuring system that transforms chemical information related to quality or quantity of analyte to useful signals. Its construction can be divided into two parts: receptor part and transducer part. Receptor part can transform the chemical information into the energy while the transducer part converts the energy to useful signal, usually electric one. Among the electrochemical sensors the biosensors should be distinguished. Their principle of operation is based on the recognition of analyte by biologically active agents such as enzymes, bacteria or antibodies [1]. Because of the low limit of detection and high sensitivity (bio)sensors are used in many areas including medicine and environmental protection.

The development of novel electrochemical sensors is combined with functionalization of electrode surface resulting in better analytical parameters of constructed sensors including limit of detection, linear range and sensitivity. The nanocomposites modifying the electrode surface should be characterized by high electrical conductivity and good mechanical resistance.

In our work we tested different composites based on TiO_2 sol, as potential matrix of (bio)sensors. The studied composites were enriched with different components including mesoporous carbon CMK-3, gold nanoparticles and Nafion. Sol-gel glasses are attractive electrode matrices as they can be easily prepared under ambient conditions, controlling pore size and geometry, they can entrapped large amount of nanocomposites, such as carbon nanotubes or metal nanoparticles, and can retain the biological activity of biosensors biomolecules [2]. Thus, the role of titania dioxide sol is to be a binding agent of modyfying layer [3]. One of the carbon material employed in construction of electrochemical sensors is mesoporous carbon CMK-3. It has a large surface area ($\sim 1000\ m^2\ g^{-1}$) and a large volume of pores ($1.3\ cm^3\ g^{-1}$), in addition it is characterized by a very good electrical conductivity [4]. Another type of nanocomponent – gold nanoparticles (AuNP) are known as excellent electrical conductor [5]. Nafion, conductive polymer, not only enhances the electrical conductivity but also improves the stability of (bio)sensors [6]. Studied modification layers were evaluated based on cyclic voltammetry redox probe performed in Fe(II)/Fe(III) solution.

2. Experimental

2.1 Reagents and samples

Titanium(IV) isopropoxide, Nafion (5% (*w/v*) solution in mixture of low aliphatic alcohol and water) and solution of $HAuCl_4 \cdot 3H_2O$ (1%) were purchased from Sigma-Aldrich (USA). KH_2PO_4, Na_2HPO_4, HCl (35%), ethanol (96%), 2-propanol, L-(+)-ascorbic acid, trisodium citrate and potassium ferrocyanide were obtained from Avantor Performance Materials Poland. HNO_3 (65%) and acetone were obtained from Lachner (Czech Republic). 0.3 mm alumina powder (used for preparing graphite electrode surface) was from Buehler Micropolish (USA). CH_3COOH (100%) and NH_3 (25% aq.) were from Merck. 0.1 M phosphate buffer solutions (PBS) of pH = 6 was prepared by mixing appropriate volumes of KH_2PO_4 and Na_2HPO_4 solutions. Distilled water from an HLP 5 system (Hydrolab, Poland) was used throughout.

TiO_2 sol-gel was synthesized by acid hydrolysis and polycondensation of the titanium(IV) isopropoxide [2]. Gold nanoparticles were synthesized during reduction of $HAuCl_4$ by sodium citrate [7].

2.2 Instrumentation

A vortex (IKA, Germany) and a Sonic 3 ultrasonic bath (Polsonic, Poland) were applied for preparation of nanocomposite for modification of graphite electrode surface. A CP-501 pH-meter (Elmetron, Poland) with a combined glass electrode ERH-11 (Hydromed, Poland) was used for pH measurements during preparation of phosphate buffer solution.

The M161 electrochemical analyzer (mtm-anko, Poland) was applied for cyclic voltammetry measurements. The experiments were carried out at scan rate of 62.5 mV s^{-1} with a three-electrode cup with the use of modified graphite electrode as the working electrode, a saturated silver/silver chloride as reference electrode and a platinum as auxiliary electrode (mtm-anko, Poland). 0.1 M phosphate buffer solution of pH = 6 was used as the supporting electrolyte.

2.3 Sensor construction

The preparation of graphite electrode surface was based on polishing it with alumina slurry and sonication in given solution (water, ethanol, nitric acid, ammonia water, L-(+)-ascorbic acid and acetone). Then, the nanocomposite consisted of titania dioxide sol, gold nanoparticles and 0.1 M phosphate buffer solution of pH = 6 mixed in appropriate proportions and 0.086 mg CMK-3 were deposited on the surface of electrode. The developed sensors were dried over saturated solution of Na_2HPO_4 mixed with water (1:1, *v/v*) and then they were kept in 0.1 M phosphate buffer solution of pH = 6 at 4°C.

2.4. Measuring procedure

The cyclic voltammetry was used as measuring technique. The standard addition method was applied as calibration method: the following portions of 0.5 M potassium ferrocyanide solution were added into three-electrode measuring cup and for each solution the voltammogram was recorded. The time between adding the portion of analyte to measuring cup and making measurement was 3 minutes.

3. Results and discussion

During the research the analytical characteristic including sensitivity towards redox probe Fe(II)/Fe(III) was tested. Based on recorded voltammograms, oxidation and reduction peak (at 320 and 100 mV, respectively) for solutions of different concentration of analyte were observed. Based on oxidation peak current the analytical characteristic including sensitivity towards Fe(II) for developed sensors was tested (Table 1). The obtained calibration curves for tested matrix nanocomposites are presented in Fig. 1. The highest sensitivity towards Fe(II) was noticed for composite consisting of 4.6 μL titanium dioxide sol,

Table 1
The examined matrices based on titanium sol-gel composites and obtained sensitivity towards Fe(II)/Fe(III) redox probe.

Type of examined matrices	Sensitivity towards Fe(II)/Fe(III) redox probe / μA mM^{-1}
unmodified graphite electrode	4.375
Nafion/TiO_2/AuNP/buffer/CMK-3 (30.1% / 22.8% / 9.5% / 37.6%)	36.257
Nafion/TiO_2/buffer/CMK-3 (25.0% / 25.0% / 50.0%)	23.237
Nafion/TiO_2/AuNP/buffer/CMK-3 (25.0% / 25.0% / 25.0% / 25.0%)	17.216
Nafion/TiO_2/AuNP/CMK-3 (33.3% / 33.3% / 33.3%)	11.949
Nafion/TiO_2/buffer/CMK-3 (25.0% / 50.0% / 25.0%)	11.899
Nafion/TiO_2/CMK-3 (33.3% / 66.6%)	3.826

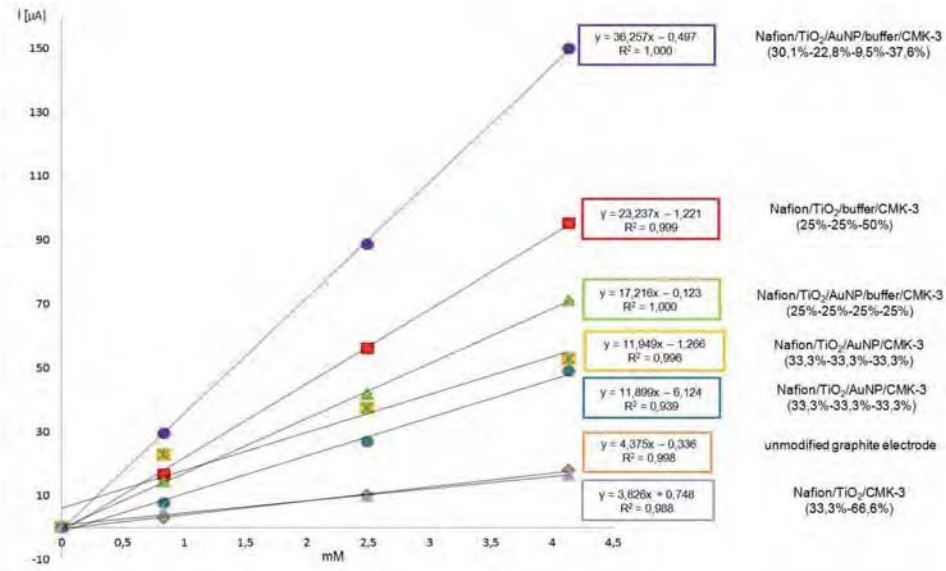

Fig. 1 Obtained calibration curves towards Fe(II)/Fe(III) redox probe for various matrix including components mixed in different volumetric ratio.

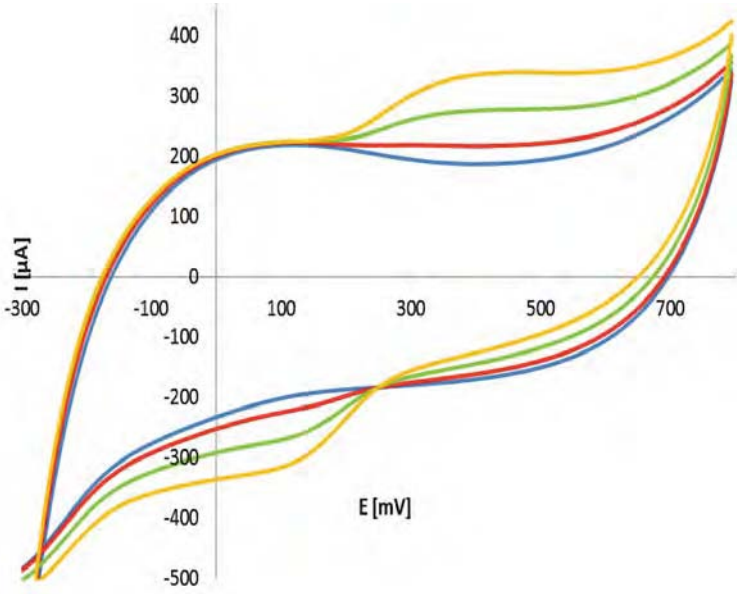

Fig. 2 Exemplary voltammograms recorded for the best nanocomposite based on Nafion/TiO_2/AuNP/buffer/CMK (30.1% / 22.8% / 9.5% / 37.6%) at scan rate of 62.5 mV s^{-1}.

1.9 µL of colloidal gold nanoparticles, 7.5 µL of phosphate buffer solution, 6.0 µL of Nafion and 0.086 g CMK-3 (in Fig. 1 marked as Nafion /TiO_2/AuNP/buffer/CMK-3 (30.1% / 22.8% / 9.5% / 37.6%)). The exemplary voltammograms recorded for the best matrix composite are shown in Fig. 2. Obtained results shows that modification of graphite electrode surface improves the properties of sensor except for sensor based on Nafion/TiO_2/CMK-3 composite.

4. Conclusions

The presented research deals with different electrode functionalizing composites based on TiO_2 sol. The studied composites consisted of titania sol modified by mesoporous carbon CMK-3, gold nanoparticles, phosphate buffer solution and Nafion mixed in different sets and volumetric ratio. The obtained results shows that electrochemical behaviour of sensors are dependent not only on the kind of nanomaterials, but also on the volumetric ratio of given components. The highest sensitivity obtained for Fe(II)/Fe(III) redox probe, was noticed for composite consisted of 4.6 µL titanium dioxide sol, 1.9 µL of colloidal gold nanoparticles, 7.5 µL of phosphate buffer solution, 6.0 µL of Nafion and 0.086 g CMK-3. The future research will be continued towards application of chosen sensor matrix for determination of antibiotic residues in environmental water samples.

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Reception and Research of New "Metallo-rubber"-based Sorption Materials

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Keywords
adsorption concentration
air analysis
dioctylphthalate
metallorubber

Abstract
In this work the possibility of use of material "metallorubber" for concentration of low-volatile organic compounds on the example of dioctylphthalate is considered. Dependences of efficiency of sorption property of metallorubber-based sorption materials on a way of processing of a adsorbed layer are received. Comparison of two types of concentration (1) the adsorption with metallorubber-based sorption materials, and (2) absorption with isopropanol is carried out. It is established that absorption concentration demonstrate erroneously low results of determination of dioctylphthalate in a gas phase in comparison with the adsorption concentration.

1. Introduction

Determination of trace contamination in air is one of the most currently important problems in analytical chemistry. For example it is necessary to determine the plasticizers in air at a concentration level of 0.1–0.5 mg m^{-3} to ensure environmental safety in the production of polymer composite materials and products based on them and to assess the safety of the using of manufactured products in everyday life. Various sorbents are widely used for carry out the concentration of organic trace contamination from gas media, but its application has some limitations. Their using is characterized by high gas-dynamic resistance of the sorbent layer which is an important deterrent when it is necessary to pass large volumes of air. Also low-volatile organic compounds difficult to desorb.

The purpose of our work was to study the possibility of using material "metallorubber" as a sorption material for concentrating of trace contamination of low volatile organic compounds. Metallorubber is block material with variable porosity. It is made by cold pressing of laid down in a certain way and dosed by the weight of a metal wire spiral. This material was developed in Samara State Aerospace University and is widely used as a damping material [1], as well as a carrier for micro- and nanoparticles of catalysts [2]. But studies of the sorption properties of this materials were not previously performed. However, it should be

Fig. 1 The photo of samples of metallorubber-based sorption materials.

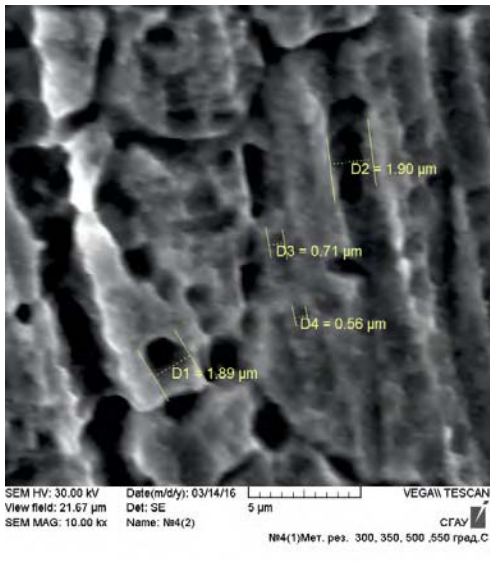

Fig. 2 The SEM image of surface of metallorubber treated with hydrochloric acid and oxidized at 700 °C.

noted that the possibility of varying the porosity and as a consequence the gas-dynamic resistance of the material layer as well as the possibility of forming oxide layers of different structures while maintaining a high specific surface area of the material makes its use as a sorption material promising.

2. Experimental

2.1 Preparation of experimental samples

Steel wire of the grade 12X18H10T was used for the production of experimental samples of metallorubber-based sorption materials. The fabrication of experimental samples consisted of the following steps:

1. To calculate of parameters of a block of metallorubber-based sorption materials on based on porosity ($\Phi = 0.7$) and surface area of a wire ($S = 2400$ mm^2).
2. To form the block of sorption material (example of sample is presented on Fig. 1).
3. To treat the surface of the block with a 20% solution of hydrochloric acid at a temperature of 70 °C for 2 minutes, to wash the sample under distilled water for removing residues of hydrochloric acid and oxidation products.
4. To form the adsorption layer by oxidizing the sample in air at temperatures of 350–700 °C for 1–3 hour (image of surface is presented on Fig. 2).
5. To wash the manufactured samples in distilled water until the end of formation of a gray precipitate.

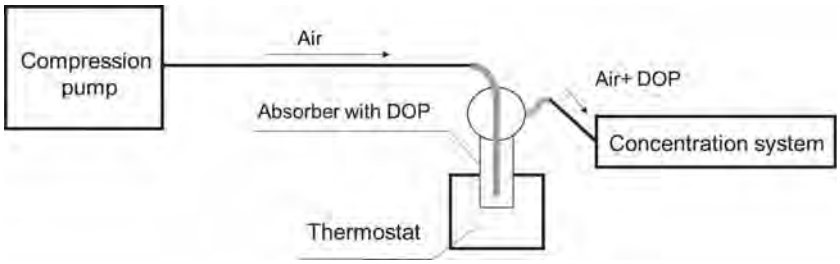

Fig. 3 The schematic diagram of experimental device for concentration dioctylphthalate from model vapor-gas mixure.

2.2 Instrumentation

It is necessary to produce model vapor-gas mixtures of dioctylphthalate in air for compare the sorption capacity of manufactured samples and also the efficiency of absorption and adsorption concentration. There are many methods for produce vapor-gas mixtures containing volatile organic and inorganic compounds, however, plasticizers, including dioctylphthalate, are characterized by low volatility, which makes it difficult to produce their vapor-gas mixtures. Experimental device (Fig. 3) was assembled for solving of this problem.

Experimental device consisted of three parts:

1. The dioctylphthalate source, which is an absorber filled with pure substance and placed in a thermostat at temperature 60 °C.
2. Concentration system.
3. Compression pump for create air flow through the absorber with dioctylphthalate and a concentration system. The air speed was 0.6 ml min^{-1}, the concentration time was 30 minutes.

In the former case an absorber filled with 5 ml isopropanol was used as the concentration system in order to carry out the absorption concentration in accordance with the standardized procedure [3]. In the latter case a cartridge containing block of metallorubber-based sorption material was used as the concentration system to carry out the adsorption concentration. The desorption of the concentrated dioctylphthalate was carried out using 2 ml isopropanol in an ultrasonic bath.

Quantitative analysis of the obtained concentrates was carried out by gas chromatography using a gas chromatograph "Crystal 5000" (ZAO SKB Khromatek, Yoshkar-Ola) with a flame ionization detector. Chromatographic analysis conditions are: quartz capillary column with SE-30 as stationary phase (10 m × 0.53 mm), column temperature 180 °C, injector temperature 270 °C, detector temperature 270 °C, flow speed of carrier gas (nitrogen) 1.8 ml min^{-1} (flow division 1:5), sample volume 3 µl.

Table 1
Weight of dioctylphthalate desorbed (m_{des}) from samples in dependence on surface treatment method of metallorubber-based sorption material.

Method	m_{des} / μg
Untreated	3.1
Treated with hydrochloric acid	31.0
Treated with hydrochloric acid and oxidized at 350°C	27.6
Treated with hydrochloric acid and oxidized at 500°C	30.2
Treated with hydrochloric acid and oxidized at 700°C	22.8

Table 2
Comparison of different methods of sample preparation in determining dioctylphthalate in air (C_{calc} is Calculated concentration of dioctylphthalate in model vapor-gas mixture).

Sample preparation method	$C_{calc.}$ / mg m^3
Absorption concentrating with isopropanol	0.4
Adsorption concentrating with untreated metallorubber-based sorption material	0.2
Adsorption concentrating with metallorubber-based sorption material treated with hydrochloric acid	1.7
Adsorption concentrating with metallorubber-based sorption material treated with hydrochloric acid and oxidized at 350 °C	1.4
Adsorption concentrating with metallorubber-based sorption material treated with hydrochloric acid and oxidized at 500 °C	1.6
Adsorption concentrating with metallorubber-based sorption material treated with hydrochloric acid and oxidized at 700 °C	1.6

3. Results and discussion

The results of estimating the amount of dioctylphthalate sorbed for various samples of metallorubber-based sorption material are shown in Table 1. The least amount of dioctylphthalate is desorbed from the untreated metallorubber-based sorption material. This is due to the fact that this sample practically does not have a highly developed adsorption layer. The amount of desorbed dioctylphthalate from samples treated at different temperatures differs insignificantly. At the same time the weight dioctylphthalate desorbed from a sample treated with hydrochloric acid, but not oxidized, is comparable with the rest. Apparently, this is due to the fact that the key factor affecting the sorption properties of metallorubber-based sorption material is the surface area, rather than its chemical composition. The aim of the etching step is to dissolve a dense oxide film that was formed on the surface of the wire as a result of its manufacture and to form a porous highly developed surface.

The application of the samples of metallorubber-based sorption material studied in the practice of the analysis air was considered. At present, absorbent

concentration with evaporation of the extract using isopropanol is used to determine the content of plasticizers in air [3]. In our work, a comparison between different methods of sample preparation was made (Table 2).

It has been shown that a standardized procedure for the determination of dioctylphthalate with absorption concentration demonstrate erroneously low results compared to adsorption concentration with metallorubber-based sorption material. This may be due to both the incomplete capture of dioctylphthalate from the model vapor-gas mixture and the addition of errors as a result of the evaporation of the extract.

4. Conclusions

Thus, as a result of the work, it has been shown that metallorubber-based sorption materials can be recommended for the determination of dioctylphthalate in air. Application of this materials will improve the accuracy of the analysis as compared to absorption concentration.

Acknowledgments

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Employment of a Prototype of Polishing Machine for Cleaning of Gold Electrode Surface for Electrochemical Application

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Keywords
cleaning procedure
gold surface
polishing machine
roughness factor

Abstract
A new polishing procedure with the use of prototype of machine to clean the surface of gold electrodes for electrochemical measurements was presented. During the mechanical cleaning of the electrodes surface, different parameters were studied, including the time and method of polishing, and electrode location. The purity of the gold surface was verified by roughness factor, which value should be in the range of 1.0–1.5 for properly prepared (cleaned) electrodes. For verification purposes the thiolation of electrode surfaces were carried out and the capacitance measurements were performed. It was proven that the employed procedure of cleaning improved the repeatability of obtained roughness factor value and influenced the further process of gold surface modification with thiols.

1. Introduction

Electrochemical sensors, due to their simplicity, sensitivity and selectivity as well as low cost and minimal requirement for sample pretreatment, constitute a good analytical tool for monitoring environmental pollutants. They also represent an instrument of green chemistry with minimal use of toxic compounds. According to the definition of the International Union of Pure and Applied Chemistry (IUPAC), a chemical sensor is a small measuring device that allows the chemical information to be transformed into analytically useful signal, most commonly electrical [1]. The electrochemical sensor consists of the receptor layer and the transducer element. The analyte interact with receptor layer in a physical, chemical or biochemical way, then the chemical information included in the sample can be converted into energy. In turn, the task of the transducer part is to convert the measured parameter to an electrical, optical or acoustic signal [2]. The scheme of chemical sensor is shown in Fig. 1.

Presently, self-assembled monolayer (SAM) – based sensors are very interesting issue for many researchers from all over the word. Sensors modification with

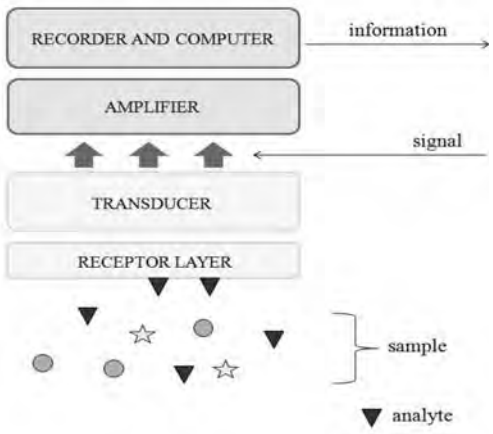

Fig. 1 Scheme of chemical sensor according to IUPAC definition [2].

the using of SAM significantly increased their applicability in the analysis of both organic and inorganic compounds, and most of all passive electrochemically ones, that could not be determined using standard electrochemical sensors. Because of its functionality, sensors based on self-organizing monolayers have been used in environmental and clinical analyzes, as well as in the monitoring of technological processes [3].

According to literature reports, an important factor in using solid electrodes is dependence of their behavior, such as reproducibility or stability, on the electrode surface conditions. Therefore, in the using of such electrodes precise and specific surface electrode pretreatment is required [4]. Contaminants such as hydrocarbons may come from the environment and adsorb non-specifically to Au surfaces making cleaning a necessary step prior to thiol monolayer formation [5]. The traditional polishing method of solid electrodes involves the drawing "eights" by hand on polishing diamonds or aluminum oxide suspensions. Reproducibility of such procedure depends on the person's manual skills, force of pressure to the electrode surface or speeds of the movements. This kind of procedure may not guarantee a repeatable cleaning method.

The most universal and well known way to verify the purity of gold is to determine the roughness factor (R_f). Its value determines the ratio between the real surface area of the metal and the geometric surface [6]

$$R_f = A_{real} / A_{geo} \quad (1)$$

where A_{real} is real electrochemical surface of gold electrode [cm^2], and A_{geo} the geometric surface of gold electrode [cm^2]. The real electrochemical surface of gold electrode can be easily determined by means of the cyclic voltammetry technique measurement in a H_2SO_4 solution. Carrying out the measurement at a known scanning rate and calculating the area of the cathodic (reduction) peak of gold

oxides, allow to calculate the real surface area of the gold electrode using the formula

$$A_{real} = red_{area} / (v \times 482) \quad (2)$$

where red_{area} is area of the reduction peak of gold oxides registered during cyclic voltammetry measurements in 0.5 M H_2SO_4 solution [μC], v is scan rate [mV s^{-1}], and 482 μC cm^{-2} is density of electrical charge for gold surface [6].

In order to consider the surface of gold to be sufficiently clean, according to the literature report, the roughness factor value between 1.0 and 1.5 should be receive. Obtaining a value higher than the specified range indicates the occurrence of cracks on metal surface, and receiving a value less than the specified range proves the presence of impurities on the gold surface. Obtaining an roughness factor between 1.0 and 1.5 is a confirmation of sufficient purity of the gold surface and allows to modifying the gold surface with using of self-assembled monolayers [6].

The main goal of presented work was development a new cleaning procedure of gold electrode surface by means of a mechanical polishing prototype. Four different procedures of electrodes cleaning were used to improving repeatability of roughness factor value. To prove the usefulness of the prototype of polishing machine it was decided to test the prepared (cleaned) electrodes in capacitance measurements. The work of a capacitive sensor is based on the electrostriction phenomenon of the thiols membranes, deposited on the gold surface electrode [7]. To confirm the purity of the gold polished by the polishing prototype, the repeatability of the capacity measurement with use of capacitive sensor were considered. Prototype of polishing machine was constructed based on an old computer disk as a tool for rotating polishing materials. Such discs guarantee a reproducible speed of rotation that can be adjusted using a specially designed electronic module. The device enables to polish two electrodes simultaneously.

2. Experimental

2.1 Chemicals

Thiol (99%) was from Sigma Aldrich (USA). Ethanol (96%), KCl, KH_2PO_4 and $Na_2HPO_4 \cdot 2H_2O$ were obtained from Avantor Performance Materials Poland. $NaNO_3$ (99.5%), HNO_3 (65%), and H_2SO_4 (95–97%) was from Merck (Germany). Diamond polishing suspensions: 3 μm was purchased Leco Corporation (USA) and 0.1 μm was from Struers (Denmark). Phosphate buffer solutions of pH = 7.4 in a concentration of 0.1 M, were prepared by mixing appropriate volumes of KH_2PO_4 and Na_2HPO_4 solutions. Ultra pure water was used throughout. All chemicals were analytical-grade reagents.

2.2 Instrumentation

Measurements by means of cyclic voltammetry were realized with using of EmStat electrochemical analyzer (PalmSens, Netherlands). All experiments were carried out with using a conventional three-electrode electrochemical cell equipped with the saturated silver/silver chloride reference electrode, a platinum wire as a counter electrode and a gold working electrode. As a supporting electrolyte 0.5 M sulphuric acid was used. The studies were performed in potential range from 0.0 to 1.4 V (*vs.* saturated Ag/AgCl) at a scan rate of 100 mV s^{-1}. During the electrochemical cleaning procedure 20 cycles of CV curves were registered.

Capacity of thiol membrane was tested by using of membrane capacitance meter CMTR-243, designed and manufactured by KSP Elektronika Laboratoryjna Polska, employed in the performed research was composed of a galvanostat, capacitance meter A/C and C/A converters. The capacitance measurements were carried out in 0.1 M phosphate buffer solution at pH = 7.4.

2.3 Tested cleaning procedures

Procedure 1: Mechanical polishing, tested under this procedure was carried out with using different grains size of diamond suspensions: 0.3 μm and 0.1 μm. Each of the electrodes was polished for 8 minutes on each polishing material, changing the electrode position by sliding in every 4 minutes.

Procedure 2: In this procedure the sonication in 0.5 M KOH solution was the first step before polishing. Polishing procedure using 0.1 μm polishing cloth for 16 minutes, with electrode position changed by sliding in every 4 minutes. Both electrodes were placed in the machine at the same time. One of the electrodes was immediately, after mechanical polishing, subjected to electrochemical cleaning using cyclic voltammetry in 0.5 M KOH solution at the potential range of –200 mV to +1100 mV and the scan rate was 50 mV s^{-1}, while the second was placed in Eppendorf with deionized water.

Procedure 3: Cleaning with using 0.1 μm polishing cloth for 16 minutes, with electrode change by sliding by hand in every 4 minutes. This procedure was used with a time shift (equal to 8 minutes) to place the electrodes in a tripod, so that the second electrode was cleaned electrochemically after the mechanical polishing.

Procedure 4: Polishing using a particle size of 0.1 μm of diamond suspension for 16 minutes while continuously changing – sliding by hand the position of the electrodes relative to the rotating polishing material. Also in this procedure, a time shift (equal to 8 minutes) of placement of the electrodes in the tripod was applied.

Examples of cyclic voltammograms registered for bare electrode in 0.5 M H_2SO_4 solution during electrochemical cleaning of electrode surface for properly and improperly cleaned electrodes, which were used to calculate the roughness factor are given in Fig. 2.

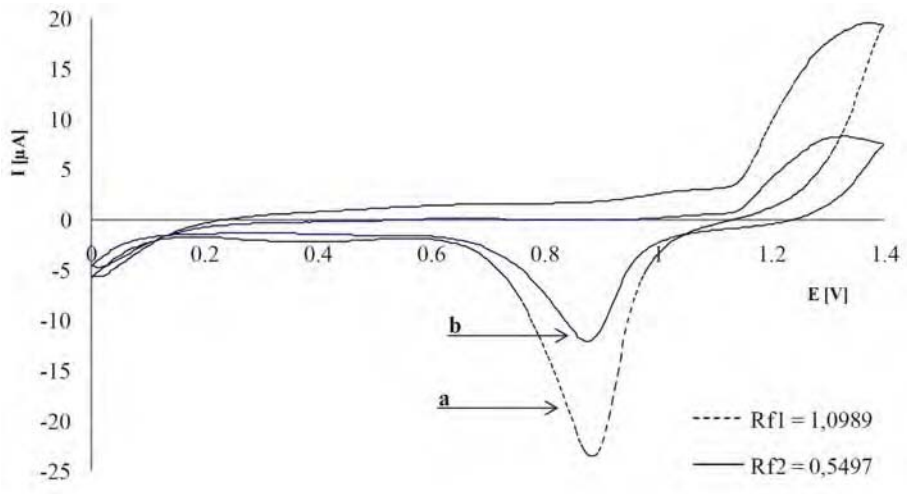

Fig. 2 Cyclic voltammograms curves registered for bare gold electrode in 0.5 M H_2SO_4 solution during electrochemical cleaning of electrode surface for (a) properly, and (b) improperly cleaned electrodes.

Table 1
Results of electrochemical cleaning of the electrodes by means of cyclic voltammetry expresed as roughness factor for four different cleaning procedures (see Chap. 2.3) for $n = 5$.

Cleaning procedure	R_f	*RSD* [%]
1	0.80	11.54
2	1.23	3.33
3	1.17	8.82
4	1.23	8.82

3. Results and discussion

The recorded cyclic voltammograms allowed us to calculate the roughness factor values, based on which we can evaluate the possibility of using a prototype of a polishing machine to prepare gold electrodes for electrochemical measurements. Table 1 summarizes the experimental results of roughness factor for the four tested procedure.

Presented results shown the quite repeatable values of roughness factor, almost for each cleaning procedure, however high values of *RSD* were obtain for some of them. The best results were obtained for the second procedure, taking into account both, the mean value of roughness factor and the relative standard deviation, which reflecting repeatability of cleaning process. The results indicate the need of using alkaline medium to remove adsorbed surfaces impurities.

To prove the usefulness of the prototype of polishing machine it was decided to test the prepared (cleaned) electrodes in a capacitance measurements. Function-

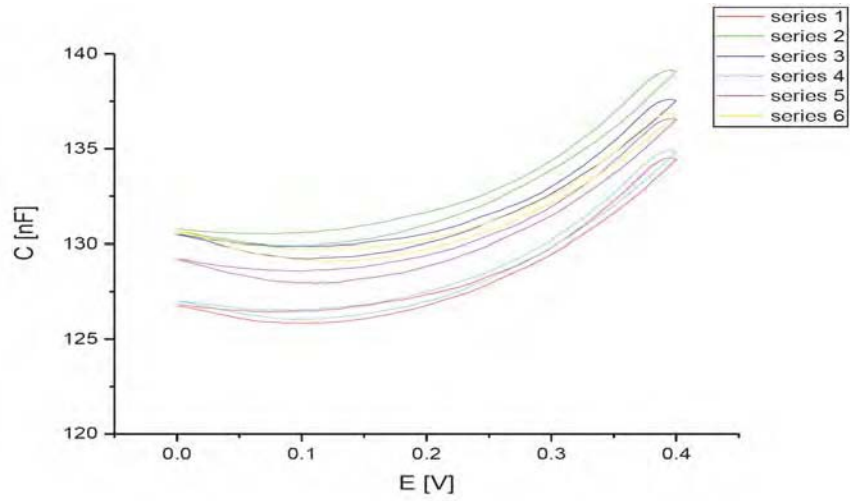

Fig. 3 Six capacitance-potential curves recorded in 0.1 M phosphate buffer solution at pH = 7.4 as a repeatability checking of gold surface modification by using of thiols (scan rate 50 mV s^{-1}).

Table 2
The results of capacitance measurements obtained for six measuring series, the capacitance value was read at potential of 200 mV.

Series	R_f	C / nF
1	1.2	131.00
2	1.1	130.04
3	1.2	127.00
4	1.2	128.84
5	1.1	129.74
6	1.2	128.22
Mean	1.17	129.14
RSD / %	3.42	1.10

ing of the capacitive sensors is based on the electrostriction phenomenon of the thiols membranes, deposited on the gold surface electrode. To modified the electrode surface 1-dodecanethiol was used. To verify the repeatability of electrode surface preparation, six series of measurements were carried out. In Fig. 3 capacitance-potential curves, recorded in 0.1 M phosphate buffer solution at pH = 7.4, are presented. For each curve the capacity value at the potential of 200 mV was read (Table 2). Repeatable capacity values proves the propriety of the second cleaning procedure by using of the prototype of mechanical polishing machine with additional step: sonication in 0.5 M KOH solution. The relative standard deviation value of capacity is slightly above 1%, what indicates really good repeatability of the cleaned surfaces of gold electrodes.

4. Conclusion

The performed study indicates that the mechanical cleaning of the gold surface is crucial step during preparation of the electrodes for measurement, when as a criterion of acceptance, the proper value of the roughness factor is considered. Especially, the second polishing procedure allowed to obtain the best results of roughness factor (1.23) and relative standard deviation of capacity measurements (3.33%). Developed polishing procedure enabled measuring of capacitive signals in supporting electrolyte with satisfactory precision.

Acknowledgments

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Stabilization of Solid Residues Obtained During Sewage Sludge Thermal Treatment

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Keywords
heavy metals
sewage sludge ash
sewage sludge dust
sewage sludge management
stabilization

Abstract
Currently wastewater treatment plants are still dealing with the problem of ecological management of sewage sludge. Nowadays, thermal utilization is considered the most eco-friendly way of processing excess sludge. However, during mentioned process, fractions of ashes and dusts are generated. Such wastes, especially dusts fraction, can be potentially harmful for the environment and wastes should be stabilized before further management. In presented work, new method of stabilization of solid residues produced during sewage sludge thermal treatment is described. Laboratory and industrial scale trails were performed. Based on the results of the conducted research it can be stated that dust and ash fraction can be stabilized together without causing threat for environment. Moreover, presented method seems to be economically justified.

1. Introduction

Nowadays the problem of sewage sludge management is becoming critical. More and more sludge is produced all over the world [1]. The main cause is rapid urbanization and vigorous development of industry [2]. Thermal utilization of excess sludge is gaining popularity, since such process leads to complete disintegration of organic pollutants. However, mentioned technique cannot be considered as a fully-complete management method, while ash and dust fractions are produced and generally landfilled [3]. Landfilling may cause threat for the environment since pollutants such as heavy metals can infiltrate to environment. There is vast variety of novel management methods, nonetheless, they do not solve the problem of incomplete waste management [4, 5]. Therefore, landfilling is becoming much more expensive, since suitable sites areas are decreasing [6].

Many researchers are seeking for new management methods. Stabilizing solid wastes with the possibility of construction material generation seems to be

especially ecologically concept because raw resources availability is decrease [7]. That is why researchers are focusing on development of artificial lightweight aggregates from wastes [8]. Cementation of produced waste with construction material production seems to be very attractive in economical point of view [9].

2. Experimental

2.1 Samples

Ash and dust fractions were obtained from two sewage sludge treatment plants located in Poland: Group Wastewater Treatment Plant in Łódź (GWT) and Wastewater Treatment Plant "Wschód" in Gdańsk (WTPW).

In both cases excess sludge is pre-dried and utilized in fluidized bed furnace. Ash and dust fractions are produced and stopped on two sets of bag filters. However, mentioned wastewater treatment plants are using different sorbents for dust fraction production. WTPW uses calcium sorbent mixed active carbon. GWTP uses active carbon mixed with sodium bicarbonate. Both facilities produce around 8 t/d of ash fraction. WTPW produce 5 t/d of dusts while GWTP produces around 1 t/d of mentioned waste. Ash and dust fraction from both sewage sludge treatment plants were sampled, transported to laboratory and stored in HDPE containers.

After that, obtained material were stabilized with proposed procedure. Ashes and dust were mixed together with cementing medium: CEM III A-S, 42,5 R and plasticizer. The amounts of certain fraction used for stabilization are described in Table 1 (next page).

Also, the industrial scale trail was performed. Ash fraction and dust fraction from WTPW was stabilized in the facility. The amounts of reagents used for stabilization single stabilization process was: 145 kg of dust, 35 kg of ash, 64 kg of cement, 181 kg of technological water and 13.5 kg of plasticizer. Several stabilizations were performed. About 9 tons of the stabilized material was produced. In Fig. 1 (next page) scheme of industrial scale trail is presented.

Appropriate amounts of samples were collected and transported to laboratory to perform leaching test and heavy metals analysis. All stabilized materials were subjected to extraction procedure to determine amounts of heavy metals which can be rinsed form obtained samples. Leaching procedure which was used for extract preparation was adopted from EN 12457-2006 *Characterization of waste. Leaching. Compliance test for leaching of granular waste materials and sludges* with following changes: liquid to solid ratio was equal 0.25 time of extraction was 72 ± 1 h; mixing with 165 rpm.

2.2 Reagents and chemicals

For mineralization presented oxidizing agent were used: 10 ml of 65% nitric acid, 2 ml of 36% hydrochloric acid, 2 ml of perhydrol. All reagents were Suprapur

Table 1
The amounts of certain ingredients used for stabilization of dust and ash fractions performed on laboratory scale (WTPW - Wastewater Treatment Plant "Wschód" in Gdańsk, GWTP - Group Wastewater Treatment Plant in Łódź).

Ash and dust fractions from	Reagents added for stabilization /g				
	Cement	Ash	Dust	Water with plasticizer	Total mass
WTPW 1	10	25	15	26	76
WTPW 2	15	25	15	30	85
WTPW 3	10	25	15	28	78
WTPW 4	20	25	15	30	90
WTPW 5	15	25	15	28	83
WTPW 6	10	25	15	27	77
WTPW 7	15	25	15	26	81
WTPW 8	10	15	25	25	75
WTPW 9	15	25	15	25	80
WTPW 10	15	25	20	30	90
WTPW 11	10	30	20	30	90
WTPW 12	15	30	20	30	95
WTPW 13	5	25	15	27	72
WTPW 14	5	25	25	27	82
GWTP 1	20	30	15	27	92
GWTP 2	10	25	10	27	72
GWTP 3	15	25	15	30	85
GWTP 4	15	30	15	30	90
GWTP 5	10	30	15	30	85
GWTP 6	10	25	15	30	80
GWTP 7	15	25	15	27	82

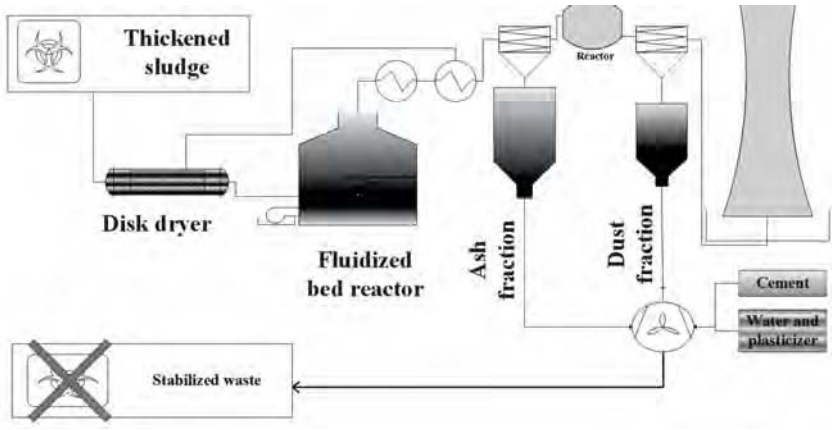

Fig. 1 The scheme of industrial scale trail.

grade and supplied by Merck. For calibration solutions preparation, extraction and leaching tests Mili-Q water was used. Calibration solutions for determination of: Cd, Co, Cu, Fe, Mn, Ni, Pb, Sb, Sn, Zn were supplied by MS-Spektrum company. All specified solutions were 1000 mg/L with varied uncertainty (from ± 4 mg/L to ± 6 mg/L), in 2% HNO_3 solution. Calibration solution for Hg determination was 100.48 ± 0.22 mg/L in 3.3% HCl, diluted in 0.001 % L-cysteine solution. Also the following modifiers for certain heavy metals determination were used: Phosphate modifier for graphite furnace AAS, $NH_4H_2PO_4$ 100 ± 2 g/L in H_2O supplied by Merck company for Sn, Ni and Cd analysis. Magnesium nitrate-palladium nitrate matrix modifier 0.2% Mg and 0.3% Pd in 1% HNO_3 supplied by MS Spektrum for Pb analysis.

2.3 Instrumentation

For mineralization Multiwave GO digestion system supplied by Anton Paar company was used. For moderate concentration heavy metals determination, Flame Atomic Absorption Spectrometer SensAA supplied by GBC Scientific Equipment (Australia) with dual beam optical system and air acetyl flame was used. Deuterium lamp for background correction and hollow-cathode lamps as radiation source were installed. For low concentration heavy metals determination, Graphite Furnace Atomic Absorption Spectrometer Savant AAZ supplied by GBC Scientific Equipment (Australia) with Zeeman background correction was used. As a carrier gas technical grade argon was supplied and hollow-cathode lamps were installed as radiation source. Mercury/MA-3000 supplied by Nippon Instruments Corporation (Japan) was used to analyse mercury by cold vapour technique and pure oxygen was used as the carrier gas. To carry out simultaneous determination of elemental species in solid samples (ashes, dust and stabilized materials) Thermo Scientific Niton Xl3t GOLDD+ XRF Analyzer was used.

3. Results and discussion

It was possible to obtain satisfying stabilization in all studied cases regardless of the ratio of reagents used. All determined heavy metals were leached from stabilized material in amount below the limit of detection in both cases, laboratory and industrial scale trails. Comparing the amount of determined heavy metals in solid stabilized material and the limit of detection in extract it can be stated that heavy metals are leached from prepared material in amounts lower than 1%. Maximum rinsing factor are presented in Table 2 (next page). All legal regulation concerning maximum allowable leaching were met.

Table 2
Maximum rinsing percentage of specified heavy metal from stabilized materials.

Element	Maximum rinsing
Cd	0.80%
Co	0.32%
Cr	0.05%
Cu	0.05%
Fe	0.00%
Hg	0.84%
Mn	0.02%
Ni	0.22%
Pb	0.20%
Sb	2.32%
Sn	0.00%
Zn	0.01%

4. Conclusions

Dust fraction obtained from WTPW and GWTP can be stabilized using commercially available cement and ash fraction produced in each of studied sewage sludge treatment plants. Such approach may lead to simplify the dust stabilization process and significant cost reductions. Since main pollutants, such as heavy metals are not rinsed form produced material, there is a possibility to produce construction materials form stabilized wastes. Proposed stabilization process can be safely implemented in both sewage sludge treatment plants while all legal standards concerning landfilling of stabilized wastes are met. However further studies have to be conducted to prove environmental safety of using stabilized wastes as construction materials.

Acknowledgments

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Characteristics of *Cucumis metuliferus*, *Actinidia deliciosa* and *Musa paradisiaca* Fragrance Profiles Using a Comprehensive Two-Dimensional Gas Chromatography with Time-of-Flight Mass Spectrometric Detection

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Keywords
Actinidia deliciosa
Cucumis metuliferus
gas chromatography
Musa paradisiaca

Abstract
The aim of the study was to characterize the headspace phase of three exotic fruits using Comprehensive Two-Dimensional Gas Chromatography with Time-of-Flight Mass Spectrometric Detection. The study was conducted on three exotic fruits *Cucumis metuliferus*, *Actinidia deliciosa*, and *Musa paradisiaca*. The scent and aroma of *Cucumis metuliferus* fruit is similar to that of *Actinidia deliciosa*, and *Musa paradisiaca*, which means that *Cucumis metuliferus* may be a pseudo-hybrid. This fact makes it possible to find similarities between these fruits. The identified chemical compounds were divided into classes, and it was found which groups of chemicals were responsible for the hedonic quality of the fruit odor and the content of the chemical compounds in the fruit volatile fraction. It has been verified which of the compounds with the highest peak area identified in the fruit *Cucumis metuliferus* are found in *Actinidia deliciosa* and *Musa paradisiaca.*

1. Introduction

In recent years, the topic of healthy food and healthy living has become very popular. For this reason, more and more scientific publications related to the analysis of food products are emerging. These publications concern the study of the characteristics of the composition of food samples and the examination of their fragrance. With the intensity of smell and hedonic quality, man can determine the nutritional value of a food product. Today, people are turning to newer, more fashionable food products. Fruits and vegetables provide the most nutritional value to a human's being life. Kiwis and bananas are one of the healthiest exotic fruits. Kiwano (*Cucumis metuliferus*), could be a very nutritious, yet not very popular, supplement to the daily human diet. *C. metuliferus* is a fruit

with green pulp with the flat, cream-colored seeds in it. The fruits are oval in shape with a length of 10 to 15 cm. They weigh about 300 grams. The fruit of the kiwano is initially green and then brightly orange with bright spots and numerous spikes [1].

Comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometric detection (GC×GC-TOF-MS) is a modern analytical technique used in many fields [2]. This technique enables an effective separation of volatile chemical compounds and allows you to identify chemical compounds from very complex matrices. With that in mind, this technique is used to analyze food samples containing large amounts of volatile organic compounds with similar physicochemical properties. By using this technique, it is possible to know the chemical composition of the headspace phase selected for fruit testing. In addition, it is possible to identify chemical compounds that affect the sensation of human senses [3].

2. Experimental

2.1 Reagents and chemicals

The fruit under study was washed under running water and then rinsed with deionized water. They were carefully picked and samples were homogenised. It consisted of cutting fruit into small cubes using a kitchen knife. Next, 5.0±0.1 g of each fruit sample was weighed and diluted with 1 ml of deionized water. Samples were placed in 20 ml volumetric flasks and sealed with silicone-PTFE membrane caps to prevent the volatile components from escaping. A sample of each fruit was made three times to make three repetitions.

2.2 Instrumentation

A two-dimensional gas chromatograph from Agilent 7980A was used for the study, which is equipped with a cryogenic modulator. This chromatograph with mass spectrometric detection incorporates in its construction the LECO Pegasus 4D fragmentation time zone analyzer. Two-dimensional gas chromatograph studies were carried out using the carrier gas as hydrogen. The first chromatographic column was filled with non-polar PDMS adsorbent and the second column filled with polar PEG adsorbent. The length of the first chromatographic column was 30 meters and the length of the second chromatography column was 10 meters. The initial temperature for the first chromatographic column was 40 °C, then the temperature gradient was applied until a set temperature of 250 °C was reached. For the second column, the starting temperature was 45 °C, followed by a temperature gradient until a set temperature of 255 °C was reached. The temperature increase in both columns was 6 °C/min. The modulation period of modulator was 4 seconds. The total analysis time was 2610 seconds.

Table 1
Distribution of volatile fractions of analysed fruits (expressed as the percentage of individual chemical groups in the volatile fraction of a fruit).

Class of compound	Content / %		
	Cucumis metuliferus	*Actinidia deliciosa*	*Musa paradisiaca*
alcohols	12.86	8.27	10.00
aldehydes	3.16	3.87	1.75
ketones	4.03	0.39	4.88
carboxylic acids	0.00	0.01	0.04
esters	54.11	72.89	72.13
terpens	13.31	2.58	0.29
hydrocarbons	12.53	11.99	10.91

3. Results and discussions

Based on the results obtained after analysis using a two-dimensional gas chromatograph, Table 1 shows the distribution of the volatile fraction as a percentage of the groups of chemicals by which perfumes are most often felt. Alcoholic, aldehyde, ester, ketone, carboxylic or terpenic hydrocarbons are characterized by the fact that the chemicals belonging to these groups produce a sense of smell and their fragrance is associated with the human being as being pleasant.

The most abundant fraction of *Cucumis metuliferus* was hydrocarbons, which are more than a half the volatile constituents. Alcohols, alcohols, terpenes and esters represented 13% of all chemical compounds. Ketones were much less than alcohol, terpenes and esters: about 4%. Aldehydes, on the other hand, accounted for 3% of all chemical compounds, and the presence of carboxylic acids was negligible.

The most abundant constituents in the *Actinidia deliciosa* fraction are esters, which constitute more than a half of the volatile constituents (72.87%). Hydrocarbon volatile fraction represented the second largest proportion of all chemicals: about 12%. Alcohol content was lower than esters and hydrocarbons: about 8%. The other groups of chemicals were less than the remaining aldehyde content of 4%, terpenes 3% of all the compounds present in the fruit volatile fraction. The content of ketones and carboxylic acids was close to zero.

The largest percentage of volatile fractions of *Musa paradisiaca* have esters of 72.13%. Hydrocarbons accounted for 10.91% of volatile chemical compounds and 10% alcohol. The percentage of ketones was about 5%. Aldehydes are a minor part of the constituents of the overlying volatiles (1.75%). Percentage of terpenes and carboxylic acids was less than 1%.

Of all the chemicals identified in the *Cucumis metuliferus* superficial phase, 31 chemicals were selected for which the chromatographic peak was the highest in the chromatogram. The Table 2 (next page) shows the analysis of presence of these compounds in the volatile fraction of fruits *A. deliciosa* and *M. paradisiaca*,

Table 2
Presentation of chemical compounds with the highest peak area identified in *Cucumis metuliferus* fruit and analyzing their presence in *Actinidia deliciosa* and *Musa paradisiaca* fruit.

Chemical compound in *C. metuliferus*	*A. deliciosa*	*M. paradisiaca*
1-hexanol	+	+
α-pinene	+	+
(*R*)-1-methyl-5-(1-methylethenyl)-cyclohexene	+	–
oxime-, methoxy-phenyl-	+	+
3-methyl-6-(1-methylethyl)-cyclohexene	+	+
α-myrcene	–	–
hexanal	+	+
6-methyl-5-hepten-2-one	+	–
hexanoic acid, methyl ester	+	+
1-methyl-4-(1-methylethyl)-cyclohexane	–	+
(*Z*)-2-hexen-1-ol	+	+
(*S*)-1-methyl-4-(1-methylethenyl)-cyclohexene	–	+
acetic acid, hexyl ester	+	+
benzoic acid, methyl ester	+	–
(+)-trans-carane	–	–
hexanoic acid, ethyl ester	+	+
1-pentanol	+	+
butanoic acid, ethyl ester	+	+
2,6-dimethyl-2,6-octadiene	–	–
α-terpinene	+	+
nonane	+	+
acetic acid, butyl ester	+	+
(*Z*)-3-hexen-1-ol	+	+
2-octanone	+	+
cis/trans-3,7-dimethyl 2-octene	+	–
1-nonanol	+	+
2-hexenal	+	+
pentanoic acid, ethyl ester	+	–
2-heptanone	+	–
butanoic acid, 2-methyl-, ethyl ester	+	+
2-nonanone	+	+

to see if the fruit of *C. metuliferus* is a pseudohybrid. Most of the identified compounds in *C. metuliferus* were identified in *A. deliciosa*, in addition to α-myrcene, 1-methyl-4-(1-methylethylcyclohexane), (*S*)-1-methyl-4-(1-methylethenyl)-cyclohexene, (+)-*trans*-carane and 2,6-dimethyl-2,6-octadiene. The other compounds testify to the human smell as sweet and fruity. Likewise, ethyl hexanoate produces a sweet, fruity odor [4]. Only 9 out of 31 chemicals found in *C. metuliferus* have been detected in *M. paradisiaca.* The fragrances of terpenes are generally associated with the scent of plants: resin, grass, forest [5].

4. Conclusions

Thanks to the use of two-dimensional gas chromatography for volatile fraction analysis of selected fruits: *Cucumis metuliferus*, *Actinidia deliciosa* and *Musa paradisiaca*, it was possible to separate and characterize the individual compounds present in fruit samples. Based on these results, it was possible to classify individual chemical compounds by their functional groups. Alcohols, aldehydes, ketones, esters, carboxylic acids and terpenes has been found in fruit samples, which are responsible for fresh and fruity scent aroma of the samples. The GC×GC-TOF-MS analysis allowed identification of certain chemical compounds in the *C. metuliferus* volatile fractions and confirmed their presence in *A. deliciosa* and *M. paradisiaca*, which means that *C. metuliferus* can be considered as a pseudohybrid.

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Evaluation of Poultry Meat Shelf Life Using Proton Transfer Reaction Mass Spectrometry

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Keywords
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Abstract
In order to safeguard the consumers' well-being, it is important to be able to quickly and accurately determine the shelf life of poultry meat. In this work, proton transfer reaction mass spectrometry with time-of-flight analysis was coupled with chemometrics to classify chicken breast samples stored under refrigeration over a period of five days. Analysis of variance was performed in order to select input data for statistical analysis. Using supervised pattern recognition algorithms, it was possible to obtain a 100% classification accuracy. Based on the results it can be concluded that proton transfer reaction mass spectrometry can be successfully used for rapid determination of poultry meat freshness and could potentially supplement the predominant methods of shelf life evaluation.

1. Introduction

Poultry is the World's most popular meat, with global consumption estimated at 13.7 kg per capita as of 2016 and is steadily increasing [1]. It is an important source of easily digestible proteins and other nutrients necessary for proper functioning of the human body. In order to preserve its wholesomeness and ensure the consumers' well-being it is vital to properly determine the meat product's shelf life. Currently, the gold standard in meat freshness assessment is the analysis of the total count of bacteria. However, this method is relatively labour-intensive and a single analysis can take up to 72 hours, which means that shelf life is determined after products leave meat processing facilities. Moreover, using total count of bacteria it is difficult to obtain information regarding the development of psychrotrophic bacteria, which proliferate during cold storage [2]. Furthermore, meat spoilage can be not only bacterial, but also chemical (e.g., auto-oxidation) [3]. An alternative approach is the measurement of the volatile fraction of a meat sample. Concentration of particular volatile indicators of meat freshness could be determined using, e.g., gas chromatography, which is however not well suited for on-line measurements [4]. Concentrations of volatile compounds in the sample's headspace can also be analysed holistically, threating their combined

aroma profile as a unique "fingerprint" which can then be used to evaluate the product's shelf life. Proton transfer reaction mass spectrometry (PTR-MS) is a particularly useful tool in said approach, especially when combined with time-of-flight analysis, as its use enables real-time quantitative determination of volatiles at low-ppb levels [5]. Combining this technique with statistical analysis methods enables meat freshness assessment without the need for qualitative analysis and sample preparation which significantly shortens the entire analysis [6].

2. Experimental

2.1 Reagents and chemicals

Fresh poultry meat was sourced from a local distribution centre in Gdańsk, Poland. Samples of homogenized chicken breast muscle (4 g) were placed in 20 ml headspace vials, covered with plastic wrap and refrigerated over a period of 5 days at 4 °C. Each consecutive day 6 samples were sealed with a cap lined with a silicon-PTFE membrane and incubated for 10 min at 30 °C in order to facilitate the transfer of volatiles to the gaseous phase.

2.2 Instrumentation

PTR TOF 1000 Ultra mass spectrometer (Ionicon, Austria) was used for headspace analysis of meat samples. Ambient air passed through activated carbon filter was used as carrier gas. Transfer line was heated to 70 °C, and mass spectra were recorded every second for a total of 120 spectra per measurement. IoniTOF v. 2.4.40 software was used to record the spectra and PTR-MS Viewer v. 3.2.3.0 to process the data. Data analysis was performed using Orange v. 3.4.3 software.

3. Results and discussion

Meat spoilage, mainly due to bacterial decomposition, leads to changes in the composition of poultry meat's volatile fraction. The research aim was to determine whether changes in concentration of particular volatile compounds in the meat samples' headspace can be used to discriminate between samples based on the storage day and thus provide tangible information regarding the product's shelf life. Averaged concentrations of compounds with particular protonated masses relative to ambient air baseline were used as input values for chemometric analysis. First, ten compounds listed in Table 1 (next page) with the highest relevance (in regard to classification) were determined using analysis of variance (ANOVA). Depicted in Fig. 1 is the change of concentration of chemical compound with $m/z = 43.05$ tentatively identified as propene over a period of five days.

Data points corresponding to relative concentration of these compounds were then normalized and analysed using principal component analysis (PCA). First

Table 1
Concentration of selected compounds listed in order of classification relevance over a period of five days.

Tentatively identified compound	Protonated mass / Da	Averaged concentration [ppb v/v]				
		Day 1	Day 2	Day 3	Day 4	Day 5
Dimethyl benzene	107.09	*n.d.*	2.4±0.7	6.2±1.2	12.5±2.7	20.9±4.8
Propene	43.05	17.3±6.3	43.1±12.9	91.9±15.5	257.3±87.3	536.9±91.5
Acetaldehyde	45.03	5.8±3.1	11.6±3.4	27.9±11.3	124.5±37.9	359.4±127.0
2-methyl-2-butene	71.09	*n.d.*	3.9±1.6	27.7±14.0	140.7±43.5	335.1±93.0
Propane	44.06	*n.d.*	1.4±0.4	2.7±0.3	6.0±1.8	11.8±1.5
Dihydrofuran / cyclobutanone	71.05	*n.d.*	3.3±1.5	27.9±15.2	149.7±41.8	338.2±110.9
Benzaldehyde	107.05	*n.d.*	2.0±0.9	5.4±1.2	11.4±2.8	18.0±5.8
Dimethyl propanol	89.10	*n.d.*	3.2±1.4	42.5±26.6	283.3±87.6	632.7±189.0
Alanine	90.05	*n.d.*	*n.d.*	2.0±1.2	10.4±2.7	22.2±7.2
Ethyl acetate	89.06	*n.d.*	3.2±1.5	44.0±23.8	296.2±86.0	642.1±217.5

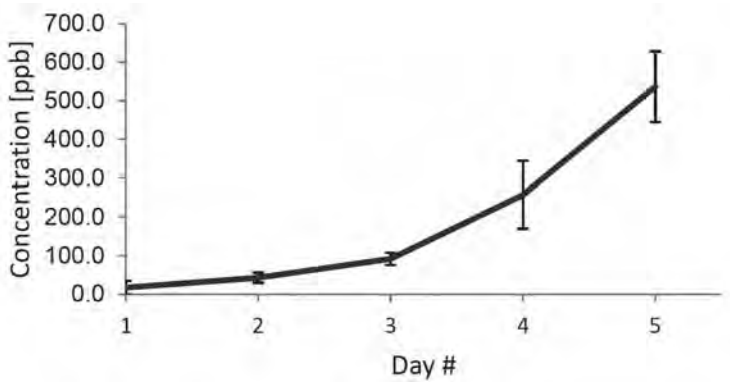

Fig. 1 Concentration of compound with m/z = 43.05 over a period of five days.

two principal components covered 99% of the variance. Data points corresponding to samples analysed after each storage day formed distinctly separate groups. A scatter plot obtained using PCA is presented in Fig. 2. It should be noted that within-group variance increased after the second day of storage which might indicate the onset of spoilage and became profound after the third day of storage. This is congruent with previously reported results [7] .

In order to develop a model for classification of poultry meat samples according to the duration of refrigerated storage three supervised pattern recognition techniques, namely *k*-nearest neighbours (*k*-NN), random forest (RF) and support vector machines (SVM) were evaluated using ten-fold cross-validation. All three algorithms allowed for classification with 100% accuracy, which was confirmed with a double-blind test in which 77% of data was used for training. All test data points were correctly classified as belonging to their respective day of storage.

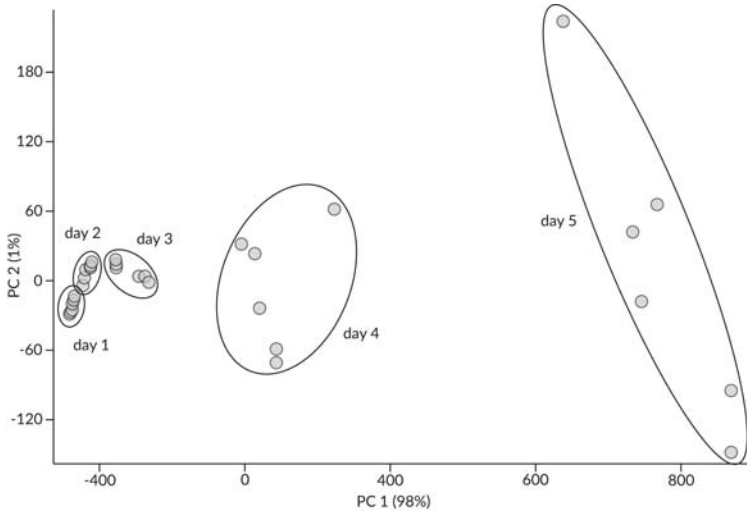

Fig. 2 Result of PCA analysis of poultry meat samples refrigerated over a period of five days.

4. Conclusions

Based on the obtained results it can be concluded that proton transfer reaction mass spectrometry coupled with statistical data analysis is a powerful technique for evaluation of poultry meat freshness. When performing holistic analysis, the determination of potential spoilage indicators using instrumental techniques can be substituted with ANOVA, in which input data most relevant for classification is automatically selected. This is made possible thanks to the high sensitivity of PTR-TOFMS. Using headspace analysis and supervised pattern recognition algorithms such as SVM it is possible successfully estimate the product's freshness without the need for sample preparation or use of solvents, conforming with guidelines of green analytical chemistry. Moreover, the time of a single analysis is no longer than several minutes, and so results can be obtained virtually immediately. Due to the possibility to easily perform quantitative analysis at single-ppb concentration levels it is possible to determine the product's shelf life with 100% accuracy given a sufficiently large training dataset.

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Direct Measurements of Odorous Volatile Organic Compounds Present in Biosolids Cakes by Proton Transfer Reaction – Mass Spectrometry Technique

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Abstract
This paper show the results of investigation of concentration of odorous chemical compounds emitted upon ageing of anaerobically stabilised biosolids that could contribute to the overall odour character of nuisance emissions. Biosolids samples from wastewater treatment plant were collected in spring time. Concentrations of volatile organic compounds using a US EPA flux hood and Proton Transfer Reaction-Time of Flight-Mass Spectrometry technique were monitored during three weeks. The concentrations of identified odorants were estimated from 0.23 ppm to 2.93 ppm. Increasing concentrations of all identified compounds were observed after 10th day from the sampling days for two samples. It can be a consequence of biological process occurring in three week time in biosolids cakes.

1. Introduction

Wastewater treatment plants are one of the most important forms of human activity, which contributes to increasing amount of chemical compounds present in atmospheric air [1]. One of the potential sources of emission from the wastewater treatment plants can be biosolids cakes, which produces many odorous chemical compounds, mainly volatile organic compounds, volatile sulphur compounds, volatile nitrogenous compounds (trimethylamine), volatile aromatic compounds (toluene, ethyl benzene, *p*-cresol, indol, skatol) or others [2]. Presence of the odour compounds emitted from wastewater treatment plants to the air has disadvantageous effect on people, including headache, concentration problems,

dizziness or malaise. Their emission has a negative effect on the plant and animal ecosystem. The level of emission of these compounds into the environment, especially volatile organic compounds, is variable. It largely depends on many factors including rate of biological changes occurring in the collected sewage, technological solutions employed at the sewage treatment plants or sewage quality [3].

One of the instrumental solutions used to determination of volatile organic compounds present in various types of samples is Proton Transfer Reaction–Mass Spectrometry (PTR-MS). This technique is based on the conversion of the neutral molecules to the ionized form as a result of the proton transfer reaction between them and hydronium ions generated inside the device in an ion source by a hollow cathode discharge on water vapour [4, 5]. In 2009 the first instruments capable of switching between various reagent ions (besides H_3O^+ reagent ions can be O_2^+, Kr^+ or NO^+) were presented [6, 7]. PTR-MS technique allows direct analysis in real time, without any sample preparation. Nowadays it is used in a wide range of scientific fields including: biotechnological application, atmospheric and environmental chemistry, medicine, food science or industrial process monitoring [7–13].

The objectives for this study are quantification and comparison of concentration of the volatile organic compounds associated with anaerobically stabilised two biosolids samples during three weeks. Differences between concentrations of odours chemical compounds with comparison to their odour descriptors can indicate which of these compounds can have the significant potential to contribute to nuisance emission from the biosolids at wastewater treatment plants.

2. Experimental

2.1 Reagents and chemicals

Approximately 10 L anaerobically stabilized dewatered biosolids samples were collected from the same location at the wastewater treatment plants "Gdańsk Wschód" located in Gdańsk, Poland in two consecutive day in spring time and transported in plastic bucket to the laboratory.

2.2 Instrumentation

Flux hood (designed according to US EPA) was used to produce emissions from the stored biosolids samples after purging (30 minutes) with a nitrogen flow of 5 L min^{-1}. After this time the biosolids samples were analysed using Proton Transfer Reaction–Time of Flight–Mass Spectrometry (PTR-TOF-MS, Ionicon Analytic, Austria). H_3O^+ ions, as the reagent ions, were produced from a pure water vapour flow in a hollow cathode discharge ion source. Volatile odours compounds from biosolids were introduced into the reaction drift tube via 1.5 m long heated

Table 1
Average concentration of odours chemical compounds present in first/second biosolids sample.

Compound	Odour descriptors	Average concentration / ppm				
		1st day	7th day	10th day	14th day	21st day
dimethyl sulfide	rubbish, seaweed, garlic, sulphur	1.31/1.29	1.42/1.28	2.01/3.77	2.12/4.11	2.93/2.76
diethyl disulfide	bad breath, garlic, sulphur	0.43/0.29	1.11/1.05	1.49/1.44	1.49/1.46	1.51/1.53
diethyl sulfide	sulphur, pungent garlic-like	0.92/0.76	0.98/0.82	1.43/1.05	1.45/1.21	1.45/1.25
dimethyl disulfide	garlic, sulphur, seaweed	1.26/1.29	1.44/1.54	2.22/3.45	2.38/3.94	2.45/3.82
toluene	solvent, gasoline, burning plastic	0.23/0.27	0.29/0.31	0.69/0.75	0.99/0.86	0.97/0.91
m-xylene	sweet, soybean sauce, cleaning product, roses, minty, solvent	0.67/0.75	1.21/1.22	1.25/1.29	1.25/1.32	1.44/1.13
α-pinene	yeast, soy sauce, sweet, pinene, floral, roses,	0.45/0.49	0.56/0.76	0.96/0.94	0.92/0.93	0.89/0.84
limonene	oranges, sweet, floral	0.45/0.43	0.65/0.49	0.92/0.89	0.96/0.92	0.94/0.92
1,3,5-trimethyl-benzene	geranium, herba-ceous, pinene, grassy	0.88/0.78	1.11/1.02	1.17/1.28	1.46/1.55	1.59/1.79
ethyl benzene	aromatic, solvent	0.75/0.72	0.94/0.79	0.99/0.88	1.45/1.49	1.55/1.42

(at 60°C) PEEK tubing with a flow rate of about 60ml min^{-1}. The key operating parameters were held at: drift tube pressure 2.0 mbar, drift voltage 600 V, temperature 60 °C and *E/N* ratio of about 130 Td (where *E* is electric field strength, *N* is gas number density, 1 Td = 10−17 V cm^2. PTR-MS measurements were conducted in a three-week period in May and June 2017 (at 1st, 7th, 10th, 14th and 21st days after sampling days, for both analysed biosolids samples). For each measurement temperature of the cakes surface was recorded. The PTR-MS instrument was calibrated by means of certified reference gases at known concentrations.

3. Results and discussion

Table 1 shows average concentration of some odorous volatile organic compounds emitted from the two biosolids samples for each measurement day. It can be observed that the highest concentrations were recorded for dimethyl sulfide and dimethyl disulfide. Other compounds emitted from the biosolids cakes revealed similar concentration at the same day. For each compound, it can be

Table 2
Odour detection tresholds (*ODT*) and odour activity values (*OAV*) for identified compounds in cakes 1.

Compound	*ODT*[a] / ppm	*OAV*				
		1st day	7th day	10th day	14th day	21st day
dimethyl sulfide	0.0030	436.7	473.3	670.0	706.7	976.7
diethyl disulfide	0.0020	215.0	555.0	745.0	745.0	755.0
diethyl sulfide	0.0033	278.8	297.0	433.3	439.4	439.4
dimethyl disulfide	0.0022	572.7	654.5	1009.1	1081.8	1113.6
toluene	0.3300	0.7	0.9	2.1	3.0	2.9
m-xylene	0.0410	16.3	29.5	30.5	30.5	35.1
α-pinene	0.0180	25.0	31.1	53.3	51.1	49.4
limonene	0.0380	11.8	17.1	24.2	25.3	24.7
1,3,5-trimethyl-benzene	0.1700	5.2	6.5	6.9	8.6	10.5
ethyl benzene	0.1700	4.4	5.5	5.8	8.5	9.1

[a] From ref. [14, 15].

observed that the highest concentration of *n* compound was between 10 and 21 days from the day of sampling of the biosolids cakes. It can be a result of biodegradation process related to microbial activity. At the 14th day from the sampling day of both cakes samples, the odour became more intensive and during last week of the measurement they were very similar. Biological process occurring in the cakes samples can have significant impact on odour type during the measurements. Commonalities between the cakes were the similar concentrations identified by PTR-TOF-MS as the cakes were aged, with a slight decline being noted at the finish of three weeks of time.

In order to determine which from the identified compounds significantly contributing to odour emissions from the biosolids cakes, odour activity value (*OAV*) was calculated, according to the following equation

$$OAV = c / c_{od} \quad (1)$$

where c is chemical concentration [ppm] and c_{od} is odour detection threshold [ppm]. Detection threshold data and calculated odour activity values are shown in Table 2.

Determination of odour activity values showed that sulphur compounds are dominated groups of identified compounds, contributing to odour emission from biosolids cakes. This fact can be related to relative small values of odour detection threshold for volatile organic compounds compared to other compounds identified in analysis samples. Chemical compounds characterized by highest values of odour activity values can be considered as potential markers of odour emission from wastewater treatment plants.

4. Conclusions

The application of PTR-TOF-MS facilitated quantification of volatile organic compounds emission from biosolids samples during three weeks and monitored their concentration during this time. Some of volatile organic compounds can be responsible for malodour. Based on the measurement with PTR-TOF-MS and calculated odour activity values, it can be observed that sulphur compounds: dimethyl sulfide, diethyl disulfide, diethyl sulfide and dimethyl disulfide can have significant impact on a summary odour character. Their exhibit varying degree of impact on odour nuisance, since they are characterised by different odour properties: odour intensity, hedonic quality or type of smell of malodorous substances. It is very important to known about properties of main odour compounds emitted from biosolids samples to elaborate effective deodorization methods.

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Voltammetric Determination of Leucovorin in Pharmaceutical Samples

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Keywords
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oxidation

Abstract
Voltammetric behavior of leucovorin has been investigated for the first time using boron-doped diamond electrode (BDDE) and method for its determination was developed. Leucovorin provides two well-developed oxidation peaks at about +900 and +1450 mV (pH = 5) on BDDE. Britton-Robinson buffer (pH = 3.0) was chosen as an optimal supporting electrolyte for its determination using differential pulse voltammetry with optimized parameters. Limit of detection 1.5×10^{-8} mol L^{-1} was reached for leucovorin determination and linear dynamic range 1.5×10^{-7}–2.5×10^{-5} mol L^{-1} was obtained. Relative standard deviation of eleven repeated measurements ($RSD_{M(11)}$ = = 0.7 %) and relative standard deviations of five repeated determinations ($RSD_{D(5)}$ < 3.0%) were calculated to confirm very good results. Applicability of the proposed method was verified by an analysis of a pharmaceutical preparation.

1. Introduction

Leucovorin (folinic acid, Fig. 1A) is one of the reduced derivatives of folic acid (Fig. 1B). Folates participate in the transfer of activated single carbon units which are involved in purine synthesis. Purines are necessary for the formation of nucleic acids, which plays significant role in cell restoration and division [1].

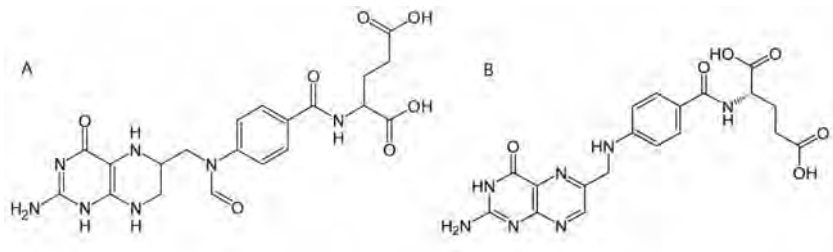

Fig. 1 Structural formula of (A) leucovorin, and (B) folic acid.

On the contrary, antifolate methotrexate blocks the purine synthesis in all cells and prohibits their further division. Due to the rapid division of cancer cells, their damage is the greatest. Therefore, methotrexate is employed as an important chemotherapeutic drug [1, 2]. Leucovorin acts as an antidote of methotrexate [3, 4]. It can be also administered in high doses as co-medication to enhance the effectiveness of the chemotherapeutic agent 5-fluorouracil in the treatment of colorectal and gastric carcinoma [5].

Although leucovorin is electrochemically active compound, only few papers dealing with its electrochemical behavior or with its voltammetric determination have been published, and, moreover, only with the use of mercury based electrodes. Allen et al. [6] described the polarographic behavior of leucovorin using dropping mercury electrode. Stejskal et al. [7] used hanging mercury drop electrode and proposed the method for leucovorin determination . Finally, Šelešovská et al. [8] described voltammetric behavior of leucovorin on mercury meniscus modified and polished silver solid amalgam electrodes and proposed the method for its determination with the limits of detection 2.2×10^{-8} and 5.0×10^{-8} mol L^{-1}.

This work represents a contribution to this area focused on voltammetric behavior of leucovorin on boron-doped diamond electrode (BDDE). This electrode was introduced for electroanalytical chemistry in the early nineties of the twentieth century [9, 10]. In terms of electroanalytical chemistry, the wide potential window about 3 V is the greatest advantage of BDDEs. Furthermore, these electrodes exhibit low noise, low adsorption on the surface, and minimal problems with passivation [11]. BDDEs are most often used in the analysis of organic compounds. Our research group has been long term interested in application possibilities of BDDE and has already published a number of papers dealing with the development of voltammetric methods for determination of various pesticides and drugs [12–17].

2. Experimental

2.1 Reagents and chemicals

The standard solution of 1×10^{-3} mol L^{-1} leucovorin (Sigma-Aldrich, Czech Republic) was prepared by dissolution of the appropriate amount in methanol (Penta, Czech Republic) and then was stored in the dark in a refrigerator. Britton-Robinson buffer of a pH value from 2 to 12 was prepared from an alkaline component of 0.2 mol L^{-1} NaOH (Lachema, Czech Republic) and an acidic component consisting of H_3PO_4, H_3BO_3 and CH_3COOH (all Lachema, Czech Republic) of the same concentration (0.04 mol L^{-1}). The electrolytes based on various concentrations of H_2SO_4 were prepared from 96% H_2SO_4 (Penta, Czech Republic). Pharmaceutical prepa-ration Leucovorin CA LACHEMA 10 originated from Pliva-Lachema, Czech Republic.

2.2 Instrumentation

All voltammetric measurements were performed by computer controlled Eco--Tribo Polarograph [18] (Polaro-Sensors, Czech Republic) with Polar.pro software (version 5.1). All measurements were provided in a three electrodes set up, where commercially purchased BDDE (7.07 mm^2) with B/C ratio during deposition step of 1000 ppm (declared by producer Windsor Scientific, United Kingdom) served as the working electrode. Saturated silver/silver chloride electrode was used as a reference and platinum wire as an auxiliary electrode (both Monokrystaly, Czech Republic). The measurements were performed at laboratory temperature (23±2 °C). Oxygen was not removed from the measured solutions.

BDDE was activated at the beginning of each working day in the solution of 0.5 mol L^{-1} H_2SO_4 by insertion of cathodic potential value of −2000 mV for 60 s and anodic potential value of +2000 mV for 60 s as well. Finally, twenty cycles from −1000 to +2000 mV (100 mV s^{-1}) were applied. Regeneration of the electrode surface between measurements was realized by insertion of potential +2000 mV for 5 s. Cyclic voltammetry (CV) was used at first for the examination of leucovorin voltammetric behavior on BDDE in dependence of pH of the supporting electrolyte and scan rate. Measurements were carried out in the potential range from −1000 mV to +2000 mV with scan rate of 100 mV s^{-1}. The influence of scan rate was recorded in the range from 25 to 500 mV s^{-1}. DPV with pulse height of +50 mV, pulse width of 20 ms, and scan rate 40 mV s^{-1} was utilized for determination of leucovorin. DPV peaks were evaluated from the straight line connecting the minima before and after the peak (tangent to the curve joining the beginning and end of a given peak).

3. Results and discussion

Voltammetric behavior of leucovorin was studied using CV. It was found that leucovorin provides two oxidation peaks in wide range of pH as it can be seen in Fig. 2. From the inserted dependence of peak height on pH, it is obvious that the highest current responses were obtained in Britton-Robinson buffer of pH = 2. On the other hand, the first leucovorin signal, which appeared to be more appropriate for analytical use, was badly repeatable in this medium (RSD_{11} = 8.5%). Therefore, Britton-Robinson buffer of pH = 3 (RSD_{11} = 1.9%) was chosen as a supporting electrolyte for the following leucovorin analyses. The linear dependence of peak height on square root of scan rate was obtained using CV for both leucovorin responses which corresponds with diffusion-controlled processes.

DPV was utilized for the development of method for leucovorin determination. Its parameters like scan rate, pulse height, and pulse width were optimized (summarized in experimental part) as well as the conditions of BDDE surface regeneration. This technique was then applied for leucovorin determination in model solutions using BDDE. An example of the obtained concentration

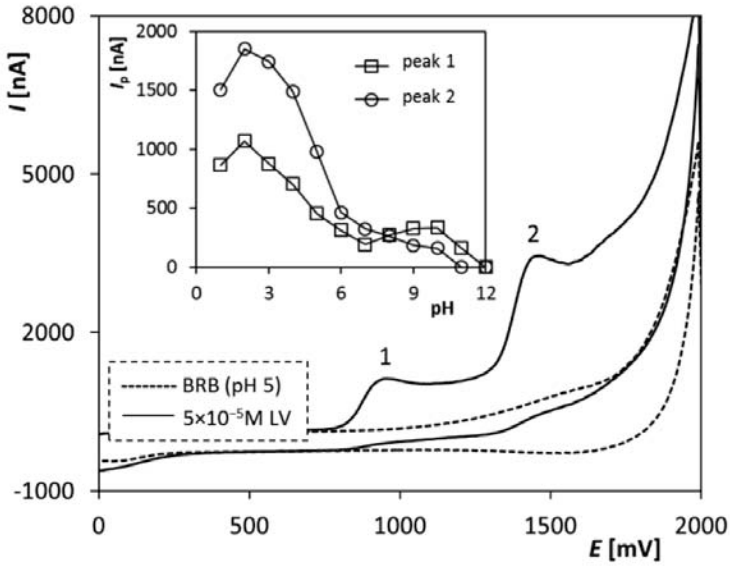

Fig. 2 Cyclic voltammogram of leucovorin obtained on BDDE. Method: Britton-Robinson buffer pH = 5.0, $E_{in} = E_{fin} = 0$ mV, $E_{switch} = +2000$ mV, $v = 100$ mV s^{-1}, c(leucovorin) = 5×10^{-5} mol L^{-1}. Inset: dependences of I_p on pH (0.05 mol L^{-1} H_2SO_4 (pH = 1), Britton-Robinson buffer pH = (2–12)).

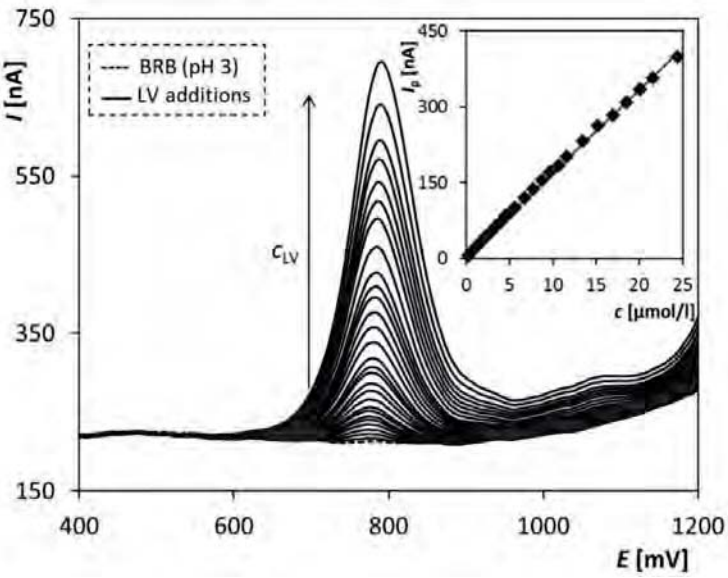

Fig. 3 DP voltammograms of leucovorin obtained on BDDE in dependence on concentration. Method: DPV, Britton-Robinson buffer pH = 3, $E_{in} = 0$ mV, $E_{fin} = 1200$ mV, $v = 40$ mV s^{-1}, pulse height = 50 mV, pulse width = 20 ms, c(leucovorin) = 2.99×10^{-7}–2.45×10^{-5} mol L^{-1}. Inset: dependence of I_p on c(leucovorin) for the peak 1.

Table 1
Results of repeated determination of leucovorin in model solutions.

Added / mol L^{-1}	Found / mol L^{-1}	Recovery / %	RSD_5 / %
1.0×10^{-5}	$(1.010\pm0.017)\times10^{-6}$	98.0–105.0	2.57
3.0×10^{-6}	$(3.030\pm0.035)\times10^{-6}$	98.3–102.6	1.74
3.0×10^{-7}	$(3.000\pm0.029)\times10^{-7}$	99.0–102.6	1.46

dependence in the range from 2.99×10^{-7} to 2.45×10^{-5} mol L^{-1} leucovorin is presented in Fig. 3. It demonstrates very wide linear dynamic range which was from 1.5×10^{-7} to 2.5×10^{-5} mol L^{-1}. The following statistical parameters of the proposed method were obtained, namely limit of detection 1.5×10^{-8} mol L^{-1} and limit of quantification 5.0×10^{-8} mol L^{-1}. The repeatability of leucovorin determination was tested by its five times repeated determinations for various concentration levels of the analyte (1.0×10^{-5}, 3.0×10^{-6}, and 3.0×10^{-7} mol L^{-1}). The obtained results summarized in Table 1 are accurate and prove very good repeatability of the proposed method ($RSD_5 < 2.6\%$).

The applicability of the developed voltammetric method was verified by analysis of the pharmaceutical sample, namely the powder for preparation of injection solution with declared content 10 mg leucovorin per vial. Injection solution was prepared by dissolving of leucovorin powder in distilled water according to the producer instruction. The content of leucovorin was determined five times in this injection solution using the standard addition method. The determined amount of 10.08±0.12 mg leucovorin per vial (recovery 98.7–102.8 %, RSD_5 = = 1.81%) coincided with the declared one. Therefore, it could be concluded that the designed method is suitable also for analysis of pharmaceutical samples without any complicated procedure only after simple dilution and it can be applied for quality control of pharmaceuticals.

4. Conclusions

Voltammetric behavior of leucovorin on BDDE using CV was described in the present paper. It was found that two anodic signals belonging to the oxidation of leucovorin could be recorded in a wide pH range. The highest stable signal was recorded in Britton-Robinson buffer of pH = 3, which was applied in all further analysis. The first oxidation signal was found suitable for analytical purposes. Thus, DPV method was successfully applied after its optimization to the analysis of model solutions as well as pharmaceutical sample.

Acknowledgments

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The Use of RP-HPLC-Q-TOF-MS as a Powerful Tool for Selection of Water Pollution Marker

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Keywords
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Abstract
Water pollution is a huge problem for everyone in the world. The use of markers of water pollution can be a universal tool for water quality control. Nowadays, indicators specific to wastewater, belonging to the group of emerging contaminants become popular. Due to their low concentration, very sensitive techniques should be applied for their determination. In this study the HPLC-Q-TOF-MS was used for the determination of the emerging contaminants that can be presented in wastewater. Moreover, two different extraction procedure and chromatographic conditions were used in order to obtain the best results. Compounds such as acesulfame-K, caffeine, and paracetamol were detected in raw wastewater sample, whereas only acesulfame-K was found in effluent sample. It seems that this compound could be selected as water pollution marker in Polish water.

1. Introduction

Water pollution is a serious worldwide problem that requires concepts or plans deriving urgent solutions. Every day, several million tons of domestic, agricultural and industrial wastes are discharged into environmental water. While water quality investigations have been generally focused on bacteria, heavy metals, nutrients and priority compounds such as persistent organic pollutants, recent research has revealed to the presence of hundreds of new organic contaminants in wastewater or impacted surface and groundwater. These compounds are classified as naturally or synthetic occurring chemicals, which have the potential to enter to natural environment and cause generally unknown adverse ecological and human health effects. Although new emerging contaminants are released to the environment for a long time, they are not commonly monitored, because their determination requires using very sensitive techniques. Moreover, their ecotoxicological effects and environmental fate are often unknown or rarely fully understood [1, 2]. Nevertheless, due to their good solubility, stability in aqueous environment, sustained release and relatively slow degradation during treatment

process, emerging contaminants, such as artificial sweeteners, caffeine, pharmaceuticals, personal care products or illicit drugs, can be considered as water pollution markers specific to wastewater [3].

The aim of this study was to evaluate extraction procedures and chromatographic methods that can be applied for the determination of selected emerging contaminants in wastewater. Potential of reversed-phase high performance liquid chromatography coupled with time-of-flight mass spectrometry (RP-HPLC-Q-TOF-MS) for the identification of emerging contaminants residue in wastewater has been established. Selection of water pollution markers specific to sewage has been proposed.

2. Experimental

2.1 Chemicals and reagents

Acesulfame-K was purchased from Nutrinova (Germany). Caffeine and paracetamol were purchased from Sigma-Aldrich (USA) and *N*-(2-methylcyclohexyl-) sulfamate, which served as internal standard, was obtained by synthesis [4]. Acetonitrile (HPLC grade), methanol (HPLC grade) and formic acid (>98%) were purchased from Merck (Germany). Ethyl acetate (LC-MS grade) and acetone (LC-MS grade) were obtained from Sigma Aldrich (USA). Methanol (LC-MS grade) and acetonitrile (LC-MS grade) were purchased from VWR Chemicals (USA). Ultrapure water was prepared using HPL5 system from Hydrolab (Poland). Ammonium (analytical grade) was purchased from Chempur (Poland).

2.2 Instrumentation

The RP-HPLC-Q-TOF-MS analyses were performed using the Agilent 1290 LC system equipped with a binary pump, an online degasser, an autosampler and a thermostated column compartment coupled with the 6540 Q-TOF-MS with a Dual ESI ion source (Agilent Technologies, USA). LiChrospher 100 RP-18e (250 × 4.6 mm, 5 μm; Merck, Germany) column was used in order to separate analytes. Two different solvent mixtures were examined and applied as a mobile phase: one method has been focused on methanol and water mixture with formic acid (0.1%, *v/v*) and the second one was prepared based on acetonitrile and water, both acidified with formic acid (0.1%, *v/v*). In both case, the gradient elution was: 5% of B in 0 min, 0–20 min linear increase from 5 to 100% of B and then 100% of B for 5 min. The last step was conditioning of the column for 5 min with 5% of B. The flow rate of mobile phase was 0.7mL/min and the injection volume was 2μL. The column temperature throughout the separation process was kept at 40 °C. The ESI source was operated with positive and negative ion mode. The fragmentor voltage was set at 100 V and the mass range was set at 100–1000 *m/z* in MS. Furthermore, nebulizer gas was set at 35 psi, capillary voltage was set at 3500 V, and drying gas

flow rate and temperature were set at 10 L/min and 300 °C, respectively. The TOF-MS system was calibrated on a daily basis.

2.3 Sampling and sample preparation

Wastewater samples were collected from local wastewater treatment plant in Swarzewo (Pomeranian Voivodeship, Poland) in May 2017. Wastewater treatment plant Swarzewo is using mechanical, chemical and activated biological treatment. This place is surrounded by tourist towns and villages and received domestic and industrial discharges, especially from food industry. All collected samples were kept in glass bottles and stored in freezer until the extraction (not longer than 48 hours).

Two different extraction produce were evaluated. They were based on the solid-phase extraction. In both cases, SPE was performed using Strata-X Polymeric RP cartridges. In the first case, 50 mL of unfiltered wastewater sample (pH ~ 8) was acidified to pH ~ 3 and the 10 µL of IS was added. In the second case, only the 10 µL of internal standard was added to the samples. Both procedures of SPE were based on literature [5, 6]. It was also decided to resign from the washing step in order not to lose the analytes. Moreover, the analytes were eluted gravitationally and the gained extracts were evaporated to dryness under the a gentle stream of nitrogen. Furtherer, the extracts were reconstructed in 1 mL of mobile phase solution. All reconstructed extracts were filtered and injected (2 µL) directly into the HPLC-Q-TOF-MS system. The scheme of both procedures is shown in Fig. 1. Meanwhile, the mixture of three standards (caffeine, acesulfame-K, paracetamol) was analyzed.

3. Results and discussion

In this presented study, the samples of raw wastewater and effluent were analyzed by SCAN mode. The identification of selected emerging contaminants was based on on-line databases and comparison of mass received during analysis of real samples and mass of standards. The whole procedure of identification compounds is shown in Fig. 2.

Compounds such as acesulfame-K, paracetamol and caffeine were detected in raw wastewater. They are daily use product ingredients, so their occurrence in domestic and industrial wastes is widespread. The results are shown in Table 1. Surprisingly, only acesulfame-K was found in effluents sample. The low intensity of chromatographic signal indicates the low concentration of this compound in the purified wastewater. Probably, this compound is partially removed during the wastewater treatment. Due to this fact, this artificial sweetener is probably being discharged with effluents to waters of Puck Bay. Therefore, it could be chosen as a marker of anthropogenic pollution of Polish waters. The obtained LC-HRMS chromatograms of acesulfame-K are shown in Fig. 3.

Comparing the two methods, method B seems to be more suitable for determination of emerging compounds, in particular artificial sweeteners. The

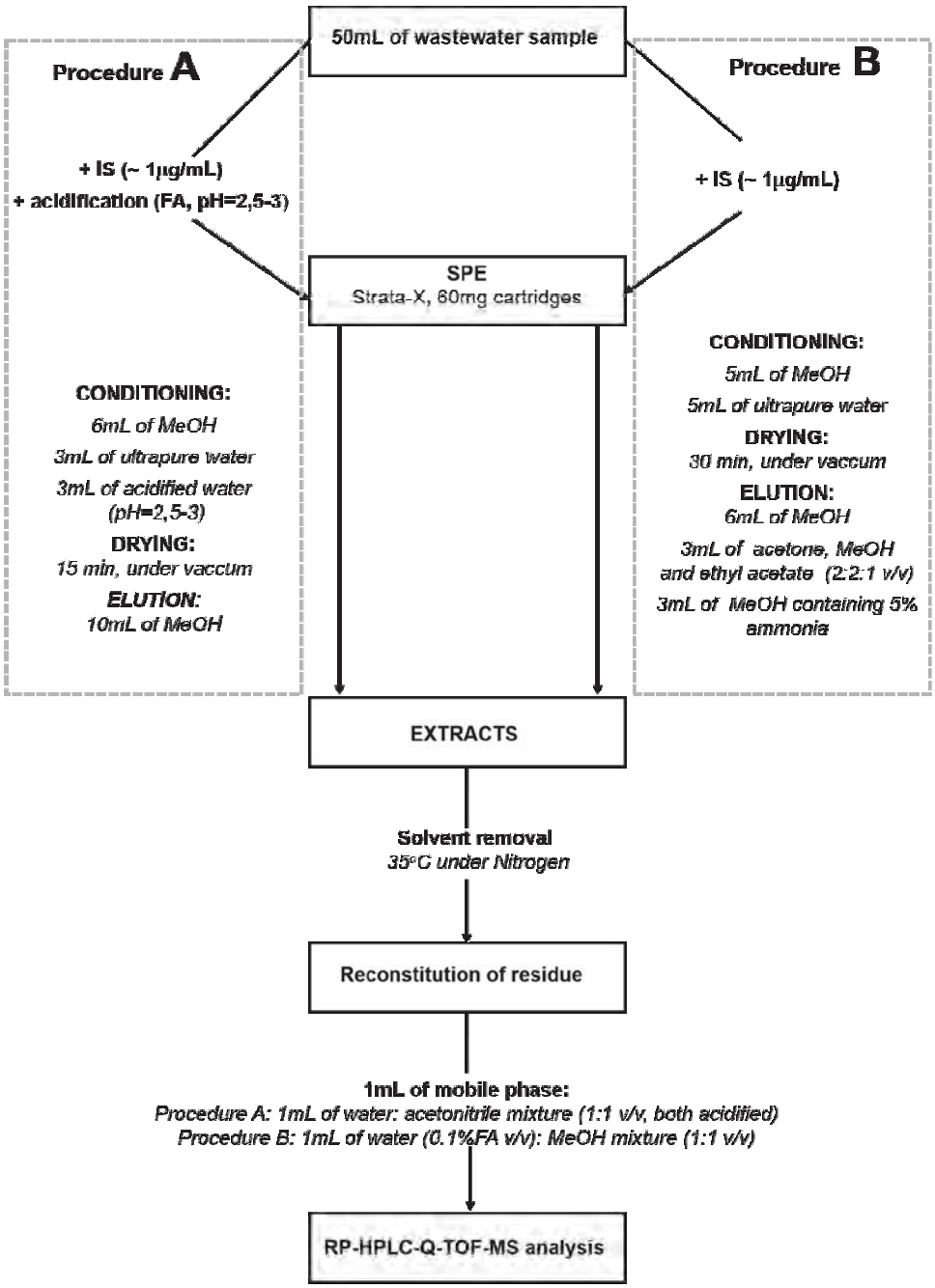

Fig. 1 Scheme of used two extraction procedures.

use of method A did not confirm the occurrence of acesulfame-K in both wastewater samples. Other analytes could be missed out too. Furthermore, the uses of methanol as the one component of mobile phase instead of acetonitrile enable to obtain better peak separation.

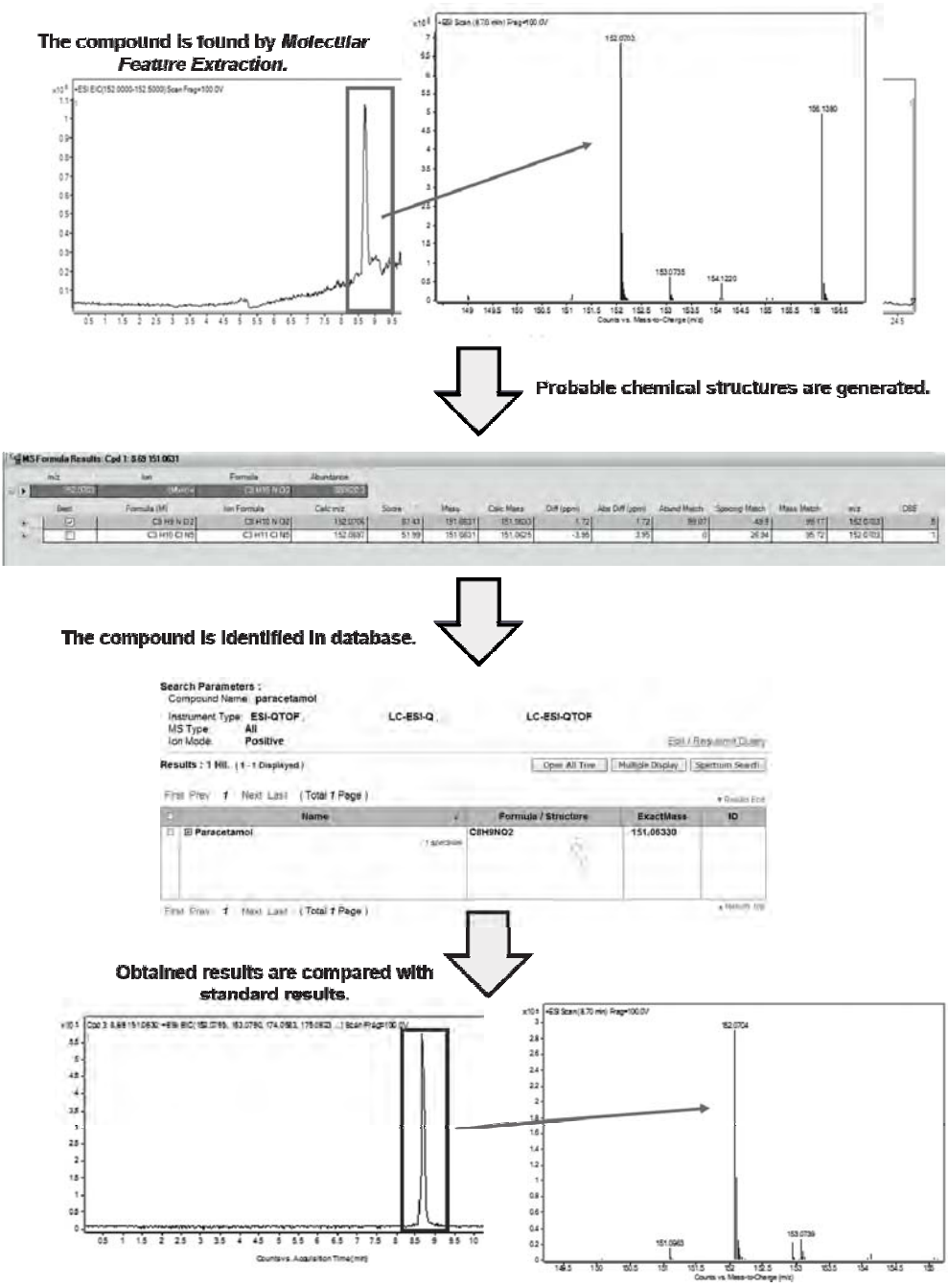

Fig. 2 Procedure of compounds identification.

Table 1
Emerging contaminants detected in raw wastewater.

Compound	*m/z* of standard ion	*m/z* of experimental ion	Mass accuracy / ppm
Acesulfame-K	161.9860	161.9856	2.43
Caffeine	195.0876	195.0872	2.05
Paracetamol	152.0704	152.0703	0.66

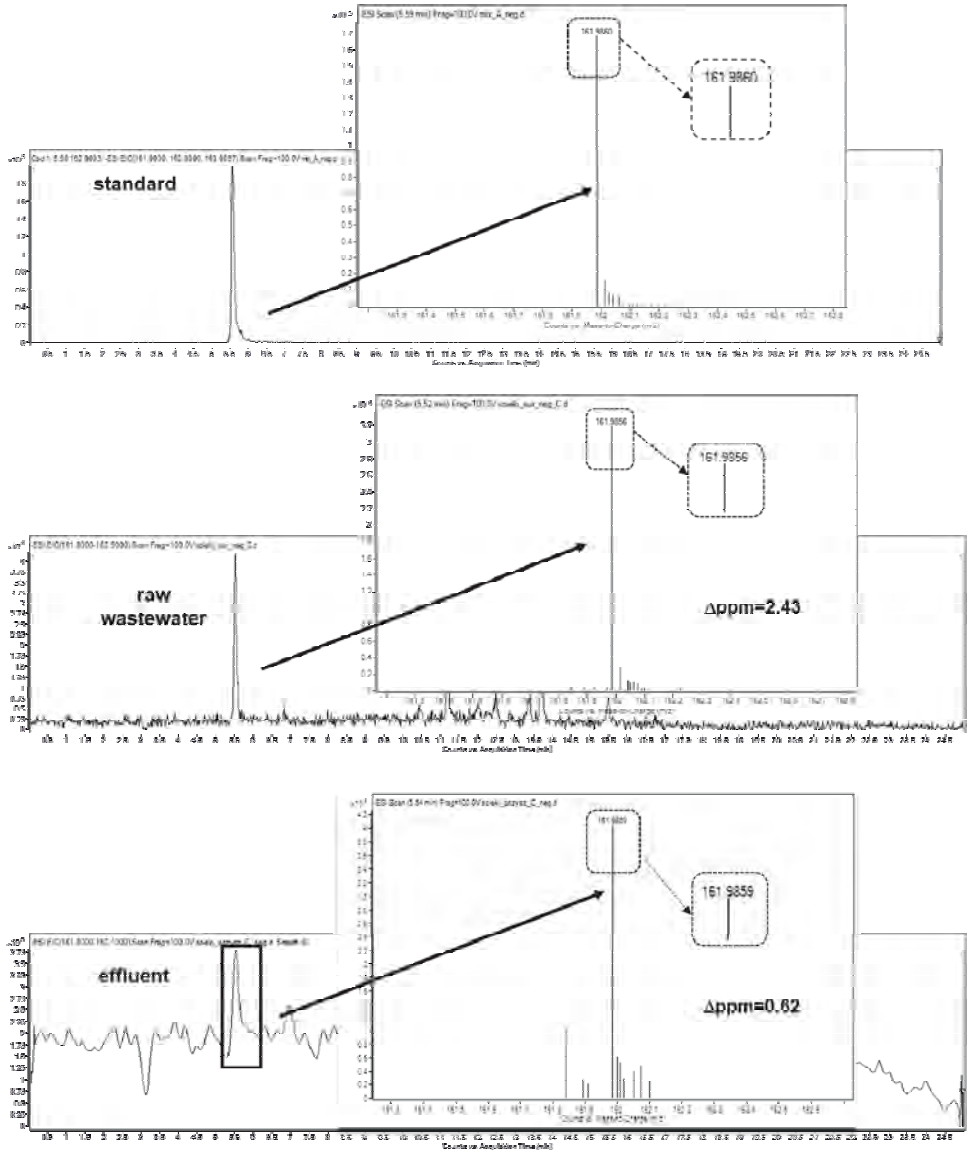

Fig. 3 Obtained LC-HRMS chromatograms of acesulfame-K.

4. Conclusion

To sum up, the solid-phase extraction with Strata-X cartridges enables the isolation of emerging contaminants from wastewater. The procedure B seems to be more suitable for extraction of acesulfame-K from wastewater sample. Results confirmed the reports that acesulfame-K is not completely removed during the treatment process and may be used as marker specific to wastewater contamination. Furthermore, detection of acesulfame-K in effluent sample proved that RP-HPLC–Q-TOF-MS technique can be successfully applied as powerful tool for selection and monitoring of water pollution marker. However, further research to detect other emerging contaminants in purified wastewater is required.

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Functionality Assessment of the Citrus Hysteria Peel as a Protective Barrier Using Gas Chromatography

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Kaffir lime
peel
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volatile fraction

Abstract
The aim of this study was to analyse the volatile fractions of fruit peel and pulp using two-dimensional gas chromatography technique coupled with mass spectrometry. The isolation of the compounds was achieved by microextraction to the stationary phase from the headspace of the sample (HS-SPME). Mass spectrometry with time-of-flight analyser (TOF-MS) was used to identify the chemical compounds. There are no reports in the literature on the use of these analytical techniques for Citrus hysteria fruit studies. It has been shown that the peel is a more aromatic part of the fruit. The potential distinguishing features of Kaffir lime have also been highlighted.

1. Introduction

Food quality directly affects the health of the consumer and determines the flavour and smell of the product. One of the most important features related to the quality of the fruit is their aroma. Fruity aroma is a complex mixture consisting of alcohols, aldehydes, ketones and terpenes. Characteristic flavour of Kaffir lime is attributable to a number of volatile compounds, mainly terpenes.

Kaffir Lime (*Citrus hysteria, Citrus hystrix*), belongs to the plants of Ruta family (*Rutaceae*). The fruit comes from South-East Asia [1]. It is cultivated mostly in Indonesia, Malaysia, the Philippines, Laos, Thailand and Vietnam [2]. There are many names of the fruits: Kaffir lime, Thai lime, Angel Wings (due to the shape of the leaves), Makrut [3]. In the Eastern culture only peel and tail of the fruit are of culinary use as components of curry paste, or popular spices. The juice and pulp are not used for cooking because of too pungent taste. However, they have many valuable properties, eg. antibacterial, antiviral, antitumor. That is why they are commonly used in the pharmaceutical industry and cosmetology [2, 4, 6].

The aim of the research was to identify and compare the content of volatile compounds present in the samples of pulp and peel of Kaffir lime fruit using two-dimensional gas chromatography technique coupled with mass spectrometry and

find the differences in the composition, which cause different taste various parts of the fruit. The bioactive volatile compounds were searched. Analysis of volatile fraction is an important part of food quality control, so that it is possible to determine its composition and identify the components that cause pleasant or unpleasant aroma.

2. Experimental

2.1 Material

Peel and pulp of Kaffir lime were analysed. The fruits have their origins in Thailand. They been bought on the floating market in the western part of Bangkok, called Taling Chan. Samples of fruits were imported to Poland in sealed plastic bags in portable fridge at between 10–15 ºC.

2.2 Instrumentation

The GC×GC system was an Agilent 6890A gas chromatograph (Agilent Technologies, USA) equipped with a split/splitless injector and a liquid nitrogen-based dual stage cryogenic modulator, coupled with Pegasus IV time-of-flight mass spectrometer (Leco, USA). Analyte identification was performed by comparing the registered spectra with the spectra contained in the library NIST 11 and Wiley and by comparing their retention times with literature values.

3. Results and discussion

About 500 chemical compounds were detected in samples of the peel and pulp of Kaffir limes by using two-dimensional gas chromatography. In addition, 200 substances were identified, of which 13 were primary components of the volatile fraction. The content of these analytes in the test samples amounted to more than 50% of all substances present in the sample's volatile fraction. Shown in Tab.1. are averaged results of the analysis of three samples of the fruit. Compound with the largest area of chromatographic peak is citronellal. Apart from citronellal among the compounds present in a large amount we may include other terpenes such as terpinene, thujene or limonene. The main chemical compounds identified in the sample of Kaffir lime are shown in the Table 1.

The content of selected classes of chemicals in samples of skin and pulp of Kaffir Lime is presented in Table 2. The composition of volatile fraction of Kaffir lime peel in all cases is characterized by a higher content of particular compounds than the composition of the volatile fraction of Kaffir lime pulp. The following data applies to all compounds detected in both samples. Comparing the content of the various classes of chemical compounds in the peel and pulp of Kaffir lime, it can be concluded that the chemical compounds of each of these classes predominantly

Table 1
The compounds identified in the volatile fraction kaffir lime using GC×GC-TOF-MS (RT_1 – first dimension retention time, RT_2 – second dimension retention time).

Chemical compound	RT_1 / s	Average RT_2 / s	Similarity	Unique mass
α-Thujene	962	2.128	899	93
α-Pinene	998	2.034	861	93
Sabinene	1050	2.097	939	93
Capmhene	1056	1.984	951	91
α-Terpinene	1134	2.044	939	93
Limonene	1170	2.026	936	93
γ-Terpinene	1272	2.156	914	93
Citronellal	1310	2.204	886	69
Terpinen-4-ol	1382	2.160	864	71
α-Terpineol	1402	2.408	790	59
β-Citronellol	1438	2.289	928	69
Citronellyl acetate	1606	2.132	916	69
β-Caryophyllene	1738	2.074	917	93

Table 2
Content of selected classes of chemicals in samples of skin and pulp of Kaffir Lime.

Class of chemicals	Content / %	
	Skin	Pulp
Carboxylic acids	88	12
Aldehydes	78	22
Ketones	74	26
Alcohols	69	31
Terpenes	66	34
Esters	54	46

occur in the peel. The peel is therefore the more aromatic part of the fruit. It is a natural barrier to protect the fruit against external factors and against the loss of many chemical compounds.

4. Conclusions

It was possible to identify the volatile organic compounds in samples of peel and pulp of Kaffir lime by using GC×GC-TOF-MS technique. In the case of peel, the major volatile chemical compounds were terpenes with citrusy aroma, such as citronellal and limonene. Peel is therefore a natural barrier to protect the fruit against loss of many chemicals. Due to the high content of volatile organic compounds, the peel of kaffir lime is commonly used as an fragrant spice. The high content of terpenes makes the fruit a rich source of nutrients. It is worth noting that transporting the fruit from Thailand did not cause loss of flavour of the fruit, and thus health-promoting properties.

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Real-Time Monitoring of Vegetable Oils' Thermal Degradation Using Proton Transfer Reaction Mass Spectrometry

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Keywords
aldehydes
edible oils
food analysis
proton transfer reaction mass spectrometry
two-dimensional gas chromatography

Abstract
The volatile aldehydes, which are generated during frying, can be harmful to human health; therefore the concentration of these compounds in frying fumes should be monitored. In addition, aldehydes are markers of oils' quality, and so it is possible to determine shelf-life or suitability for frying of these oils. Commonly, in order to determine aldehydes concentration gas chromatography is performed, however it does not allow for real-time analysis. The aim of this work was to present the possibility of using proton transfer reaction mass spectrometry to monitor the concentration of volatile aldehydes. As a complementary technique, two-dimensional gas chromatography was used to identify the compounds. Additionally, a hierarchy cluster analysis was performed to determine the intensity of the oils oxidation.

1. Introduction

As a result of the oxidation which occurs during frying, aldehydes are formed. They are one of the most important products of this reaction. The aldehydes vary in carbon chain lengths, degree of saturation or presence of other functional groups. Because of the molecular weight of the aldehydes, they can be divided into those in which the carbonyl group is attached to the short carbon chain and those in which the functional group is bound with the triacylglycerol fragment [1]. Short chain aldehydes can be found in any kind of oil under different frying conditions, e.g., different temperatures or frying duration [2]. Low molecular weight aldehydes may be toxic to humans [3].

Presently, to determine concentration of volatile aldehydes gas chromatography is used. The standard oil sample preparation procedure may include distillation or extraction preceded by distillation [4, 5]. However, because of its advantages, solid phase microextraction (SPME) is increasingly frequently performed [6].

Since the volatile fraction of some vegetable oils is a complex matrix, it is necessary to improve the resolution of the gas chromatography. Accordingly, it is possible to use gas chromatography modified by using two chromatographic columns connected in series. This solution is called two-dimensional gas chromatography (GC×GC). Analytes leaving the first chromatography column are introduced to the second via a modulator [7]. By coupling GC×GC with a mass spectrometer with time-of-flight analyser, it is possible to perform the analysis with excellent separation and identification of volatiles. However, this technique is not commonly used in the analysis of edible oils [8]. The use of gas chromatography unfortunately involves sample preparation step and the need to use solvents for the determination of analytes. In addition, GC cannot be used in real-time monitoring of processes such as frying.

Therefore, the alternative solution may be the use of proton transfer reaction mass spectrometry (PTR-MS). With this technique, it is possible to determine the concentration of compounds during the measurement and the measurement itself can be performed continuously throughout the experiment. Using the time-of-flight analyser, it is possible to identify and determine the concentration of most of the volatile organic compounds present in sample's headspace [9]. Nevertheless, the compound identification capability is limited, because proton transfer reaction does not involve the fragmentation of the molecule. Presented in this work is the use of GC×GC and PTR-MS as complementary methods for identification and monitoring of aldehydes which are the products of thermal oxidation of oils.

2. Experimental

2.1 Reagents and chemicals

Refined vegetable oils, namely sunflower, rapeseed and olive oil were purchased at local distribution centres in Gdansk. Aliquots of five grams of each oil were placed into 20 ml glass vials and sealed with an open top closure with PTFE/silicone septa. Subsequently, prepared samples were incubated at following conditions. For GC×GC-TOFMS analysis samples were heated for 24 hours at five different temperatures, namely 20 °C, 60 °C, 100 °C, 140 °C and 180 °C. This caused the samples to differ in degree of thermal degradation, and thus in degree of oxidation. In turn, in order to monitor the composition of the volatile fraction of oils using PTR-TOFMS, vials were placed in a custom heating device. They were then heated continuously from 20 °C to 180 °C for 15 minutes and then held for another hour.

2.2 Instrumentation

Two-dimensional gas chromatograph Agilent 7890A (Agilent Technologies, USA) equipped with a liquid nitrogen-based dual stage cryogenic modulator was used

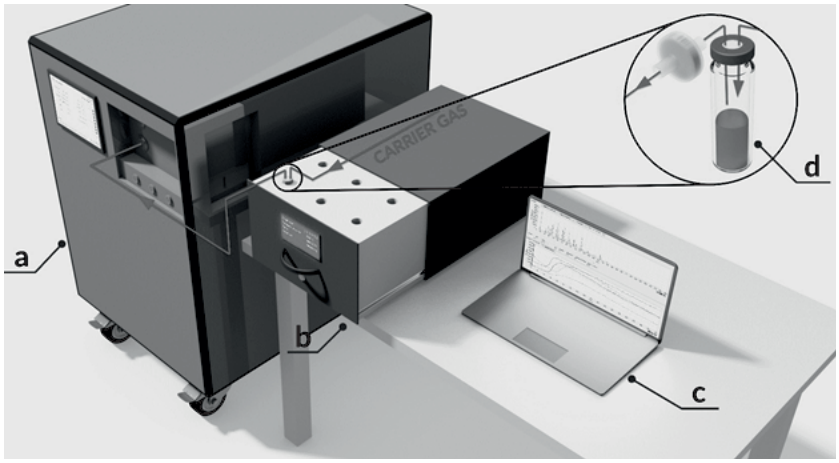

Fig. 1 Experimental setup comprised of (a) PTR-TOFMS, (b) custom heater/incubator, (c) personal com-puter with data processing software, and (d) headspace vial with a syringe filter.

to identify volatile aldehydes formed during thermal degradation of vegetable oils. Pegasus 4D (Leco, USA) mass time-of-flight spectrometer was the detector for GC×GC analysis. Hydrogen was used as carrier gas. SPME technique was performed to extract and enrich the analytes.

For real-time monitoring of aldehyde concentrations, proton reaction mass spectrometry with time-of-flight analyser PTR TOF 1000 Ultra (Ionicon GmbH, Innsbruck, Austria) was utilized. The sampling system (Fig. 1) included passing air through the filter and delivering it to the oil vial. The sample's headspace was driven dynamically via a transfer line to the PTR-TOFMS. A nylon syringe filter was used to stop oil droplets that could have formed during the process. The measurement was performed in real-time during the incubation of the vegetable oil sample and the mass spectra were recorded every 10 seconds.

3. Results and discussion

The subjects of researches were aliphatic aldehydes from C1 to C8 formed during the heating of vegetable oil. GC×GC-TOFMS was used to identify these aldehydes. Since two chromatography columns characterised by different polarity were employed, the system was characterized by very good resolution. It was possible to apply a short temperature program, which enables separation of all aldehydes in less than 20 minutes. Identification of aldehydes was performed according to the comparison of mass spectra obtained with the NIST spectral library. Therefore, as the analysed mass spectra were above 40 amu, it was not possible to identify formaldehyde in the oils' headspace. Selected aldehydes with their retention time from both columns are listed in Table 1. Retention times are given for rapeseed oil samples.

Table 1
Concentrations of monitored volatile aldehydes in oils fumes during heating.

Compound	Protonated mass / Da	RT_1 / s; RT_2 /s	Maximal concentration (concentration in 20 °C) / ppm_v		
			Sunflower oil	Rapeseed oil	Olive oil
Formaldehyde	31.018	*n.d.*	1.872±0.035 (0.0098±0.0010)	1.386±0.011 (0.0292±0.0014)	0.689±0.013 (0.0251±0.0010)
Acetaldehyde	45.033	198; 1.480	29.29±0.57 (0.259±0.074)	12.02±0.35 (0.208±0.015)	11.92±0.20 (0.0717±0.0019)
Propanal	59.049	216; 1.688	11.05±0.20 (0.482±0.046)	7.29±0.18 (0.0733±0.0056)	14.46±0.18 (2.024±0.053)
Butanal	73.065	270; 2.016	13.97±0.35 (0.0129±0.0012)	7.58±0.23 (0.202±0.025)	6.66±0.12 (0.01743±0.00096)
Pentanal	87.080	384; 3.344	11.94±0.25 (0.010±0.0053)	2.461±0.075 (0.0813±0.0077)	3.000±0.055 (0.0332±0.0021)
Hexanal	101.096	540; 2.392	14.43±0.29 (0.0032±0.0015)	2.488±0.064 (0.131±0.012)	3.133±0.055 (0.0293±0.0019)
Heptanal	115.111	750; 2.552	7.58±0.17 (0.00134±0.00067)	1.698±0.054 (0.0964±0.0081)	1.559±0.025 (0.0150±0.0011)
Octanal	129.127	960; 2.288	3.767±0.085 (0.00100±0.00047)	1.374±0.055 (0.0678±0.0062)	1.481±0.024 (0.01207±0.00094)

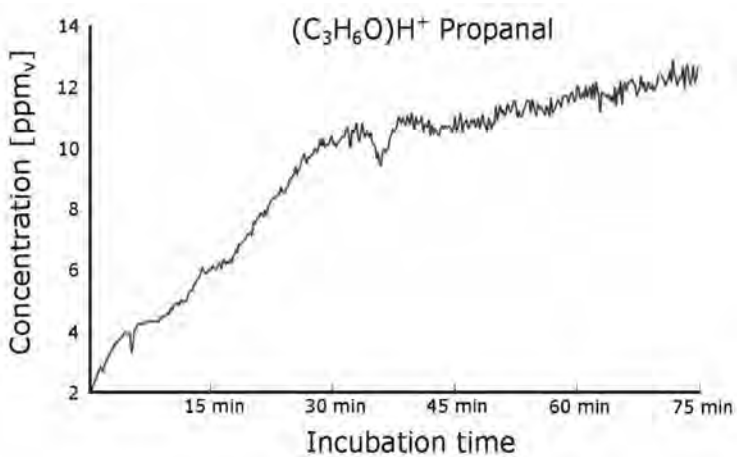

Fig. 2 Monitored concentration of propanal in olive oil fumes during incubation.

Subsequently, PTR-TOFMS was used for real-time monitoring of aldehydes generation during oxidation. Fig. 2 shows an example of a propanal concentration changes in olive oil's fumes during the incubation. It could be noticed, that the most intense increase in concentration was recorded for the oil sample heating stage.

Changes in the concentration of all monitored aldehydes are shown in Table 1. It can be seen that the concentration of aldehydes in the gas phase of the oils before incubation was below 1 ppm. This may indicate that these oils were not oxidized. Over time, the concentration of each aldehyde increased. The highest concentration changes were monitored for acetaldehyde, propanal and butanal. It should be noted that the highest concentrations of aldehydes were observed for sunflower oil. The lowest amount of these aldehydes was for refined olive oil. It can be assumed that sunflower oil is the least thermally stable.

The hierarchical cluster analysis was used to classify the degree of thermal degradation of oils according to the composition of their volatile fraction. The input data for the statistical analysis was aldehyde concentrations in the gas phase of the oils measured at the start of the experiment and after 5, 30, 45, 60, and 75 minutes of incubation. The aim of the analysis was to demonstrate that, regardless of the botanical origin of oil, it is possible to distinguish their quality. Fig. 3 shows the results of hierarchical cluster analysis with Ward's linkage method. It could be noticed that it is possible to assess the degree of oxidation of oils. The composition of the volatiles for samples at the beginning and after 15 minutes forming a single cluster. This may mean that they have a similar degree of oxidation. A separate cluster was obtained for data recorded after 30 minutes of the experiment. After 45 minutes of incubation, each tested vegetable oil exhibits the highest degree of oxidation. Data for 45, 60, and 75 minutes are located in a single cluster.

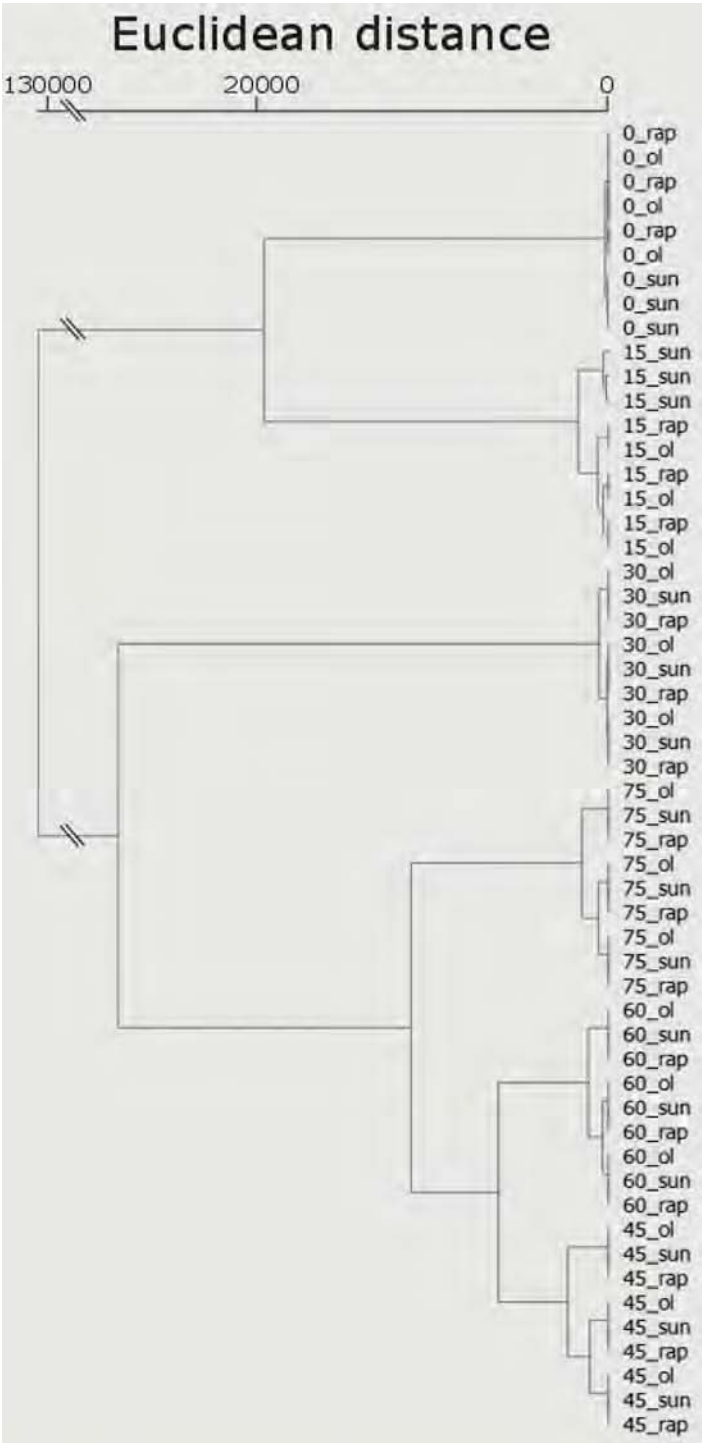

Fig. 3 Classification of oils according to their oxidation degree using hierarchical cluster analysis method.

4. Conclusions

Presented in this work is a new procedure for determining the aldehyde concentration arising from the oxidation of vegetable oils. Two-dimensional gas chromatography was used to identify these aldehydes. Real-time monitoring was performed using proton transfer mass spectrometer. The concentration of volatile aldehydes from C1 to C8 was determined. It was noted that the concentration in the oil vapours of some aldehydes exceeds 10 ppm. It should be assumed that working in rooms where these vegetable oils are used for frying may adversely affect the health of staff. The most thermally stable oil was olive oil and the least thermally stable was sunflower oil. Using hierarchical cluster analysis, it was possible to distinguish the oxidation degree of oils regardless to their botanical origin. It has been shown that each of the tested oils can be regarded as oxidised after 45 minutes of incubation. In the near future, authors plan to conduct a similar experiment in real frying conditions.

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Distinction of Citrus Fruits Based on Their Volatile Composition Using the Electronic Nose

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Keywords
citrus fruits
electronic nose
volatile organic compounds

Abstract
The aim of the study was to analyze the volatile fraction of the citrus fruit using an electronic nose device integrated with ultrafast gas chromatography. The subjects were *Citrus aurantifolia*, *Fortunella margarita* Swingle and *Citrus × floridana*. The chromatographic data obtained were analyzed by means of chemometric methods: Principle Component Analysis and Statistical Quality Control. On the basis of the charts obtained, it is possible to distinguish the fruits tested on the basis of their botanical origin and to determine the differences between the individual fruit samples.

1. Introduction

The electronic nose is a device used to perform instrumental analysis of volatile organic compounds. It is equivalent to the human sense of smell in which receptor cells have been replaced by a set of chemical sensors that are selective to the chemical groups concerned, and nerve processing by computer and software. The advantage of using the electronic nose is the short time of analysis, which results in the called "fingerprint" corresponding to the analyzed volatile fraction without information about its chemical composition.

The technology of instrumental methods development enabled the use of rapid gas chromatography in electronic noses. This allows you to obtain both quantitative and qualitative information about the analyzed chemicals. In such a system, the sensors were replaced by flame ionization detectors [1, 2]. Obtained as a result of the chromatographic analysis data is subjected to a chemometric analysis. The most commonly used are principal component analysis and statistical quality control analysis. Analysis of the main components is a technique of dimension reduction, based on the creation of several new variables in the linear combination of the original variables. New variables, called main components, are presented in the form of a matrix, containing data on the number of samples and the number of measured parameters. For highly coinial replacement data, some main components retain the same information as the primary

variables. This makes it easy to place samples and variables and visual analysis. The PCA technique is a relatively simple graphical interpretation of data, so it is often used prior to classification using more complex methods. The result of the analysis of the main components is a graph showing the objects in the group. Objects are grouped in such a way that "similar to each other" is assigned to the same group. It follows that objects in one focus are homogeneous with regard to certain characteristics, defining the focus [3].

SQC analysis was used to control the quality of fruit samples. The analysis makes it possible to distinguish between samples. This is a quick and easy-to-use method to isolate the differences in the sample group. SQC is a statistical evaluation of the occurrence of sample differences in a given batch of samples. Based on the results of the SQC analysis, the sample can be eliminated if it is significantly different from the other samples in the group [4].

In this paper, lime, kumquat, and limequat were analyzed. Limequat is a fruity hybrid that will grow through the intersection of lime and kumquat. The limequat looks and smells like lime, so the fruit is a suitable test material for distinguishing fruits using the electronic nose.

2. Experimental

2.1 Sample preparation

Twenty-ml vials were used for the analysis. The vials were washed in cold water and then rinsed five times with deionized water. The fruits of kumquat, lime, and limequat are also washed under cold running water, rinsed with deionized water and cut into small pieces. The tools used to homogenize the fruit were washed twice with deionized water. Next, fruit pulp was placed in vials, weighing 5.0 ± 0.1 g. The vials were sealed using aluminum tips with silicon-teflon membrane.

2.2 Instrumentation

The analysis of the volatile fraction of kumquat, lime and lucid fruit was conducted using the electronic nose of Alpha M. O. S. of the trade name Heracles II. The electronic nose is integrated with ultrafast gas chromatography with a set of independent column chromatography. The columns have a length of 10 m and differ between polarities (nonpolar MXT-5 and medium polar MXT-1701). The electronic nose also features an HS-100 automatic sample feeder, a dispenser for both gaseous and liquid samples, and a sorption trap. An electronic nose to identify analytes uses two flame ionisation detectors. The inherent element of the device is AlphaSoft V12 software and Arochembase V4 library, allowing characterizing the identified chemical compounds and their sensory evaluation.

Table 1 contains parameters for analysis of volatile fractions of kumquat, lime and lime fruits using Alpha M. S. Heracles II

Table 1
Conditions of analysis using Alpha M. O. S. Heracles II.

Element	Operation	Parameter
Autosampler HS-100	Drying	Thermostat temperature: 80 °C Thermostat time: 300 s
	Rinsing	Rinse time: 90 s
	Mixing	Mixing speed: 500 rpm
Heracles II	Introduction of the sample to the dispenser	Volume of the gas sample: 2500 µl Sample rate: 250 µl s^{-1} Carrier gas pressure: 250 kPa Volumetric carrier gas intensity: 500 µl s^{-1} Temperature of the dispenser: 200 °C Time to put the sample into the sorption trap: 15 s
	Adsorption / desorption of analytes	Starting temperature of the sorption trap: 40 °C Initial pressure in the sorption trap: 80 kPa Time of desorption of analytes: 20 s
	Analysis	Temperature program: 40 °C for 2 s, rise 3 °C/s to 270 °C for 18 s Carrier gas: hydrogen
	Identification of analytes	Detector temperature: 270 °C

3. Results and discussion

The result of the analysis of the main components is a graph showing the objects in the group. Objects are grouped in such a way that "similar to each other" is assigned to the same group. It follows that objects in one focus are homogeneous with regard to certain characteristics, defining the focus [3]. As a result of the analysis of the main components for the interpretation of the data obtained using the electronic nose, the graph is shown in Fig. 1. Cluster analysis allowed classification of fruit samples into groups based on the similarity of composition of their volatile fractions. This resulted in three groups: lime fruit, kumquat and lime. Created clusters are clearly separate from each other, which confirms that these are different fruits. Distribution of groups at equal distances from each other may indicate that the fruit contains similar content and amount of chemical compounds. It can also be concluded that the volatile fraction of limequat, which is a hybrid of kumquat and lime fruit, contains both the chemical compounds present in one and the other in similar concentrations of the parent fruit.

Using the SQC analysis, the graph shown in Fig. 2 is obtained. Samples of fruit belonging to one group should be in one area. In Fig. 2 we observe that the differences between the points corresponding to kumquat fruit samples are small. A slight divergence of points was also observed for lime fruit samples. However, the differences between the points belonging to the group of lime fruits are more noticeable. Points corresponding to samples of lime fruits are not in line and the area occupied by them is much larger than that of kumquats and limes. This

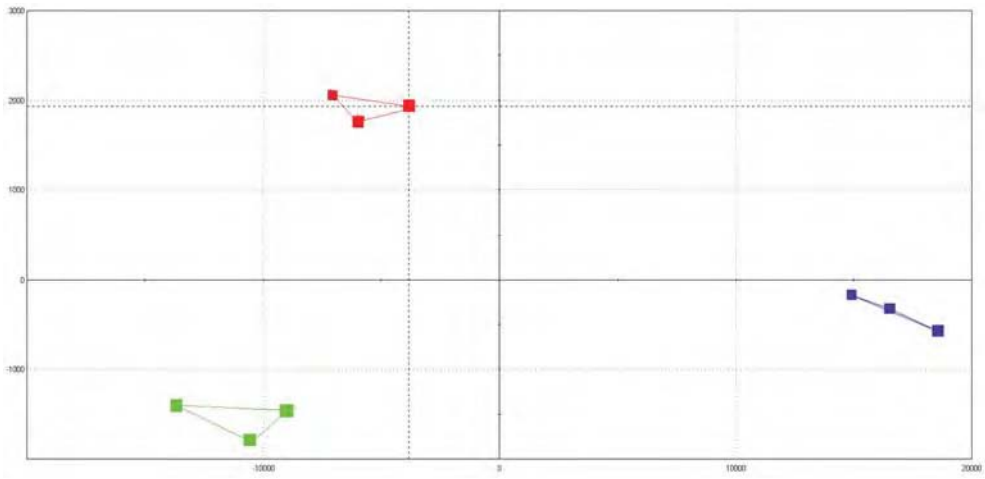

Fig. 1 PCA analysis of fruit samples (*Fortunella margarita* Swingle, *Citrus aurantifolia*, *Citrus × floridana*) using Alpha M. S. Heracles II.

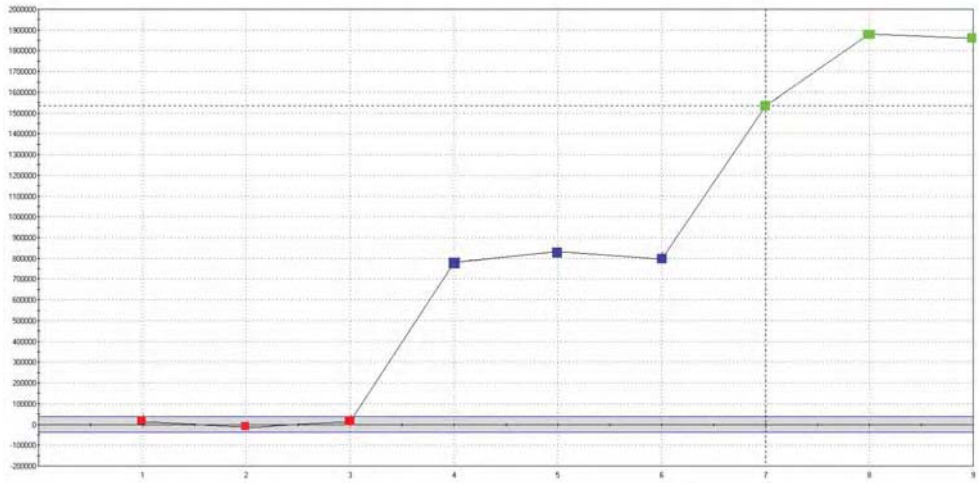

Fig. 2 Results of the SQC analysis with the reference group of fruit of the kumquat derived from the analysis of fruit samples (C*itrus aurantifolia*, *Fortunella margarita* Swingle, *Citrus × floridana*) using Alpha M. S. Heracles II.

proves that there is a greater difference between the samples and thus a greater tolerance for the whole group.

4. Conclusions

The electronic nose, designed as an instrument for imitating human perception of smell, is an innovative measurement system for volatile organic compounds. As

a non-invasive technique, e-nose combined with chemometric methods is a popular system used in food quality control. The advantage of using an electronic nose is simplification of measurement procedure, small amount of sample used, no chemical reagents required and high sensitivity and low measurement cost. For this reason, the electronic nose is used for studies on the period of validity, maturity of vegetables and fruits, monitoring of production processes and evaluation of the authenticity of products.

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Detection of Apple in Orange Juice Using Ultra-Fast Gas Chromatography

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Keywords
food authentication
fruit juices
ultra-fast gas chromatography

Abstract
The determination of authenticity is an increasingly important issue for food quality and safety. The use of an electronic nose based on ultra-fast gas chromatography technique ensures rapid analysis of the volatile compounds from food products. Due to the fact that this technique enables chemical profiling of agricultural products, it can be an effective tool for authentication when combined with chemometrics. In this article presented is a methodology for classifying NFC (Not From Concentrate) juices. Samples tested were: pure orange juice, pure apple juice, as well as mixtures of these juices with known percentage of base juices. Classification of juice samples was carried out using chemometric method - Principal Component Analysis (PCA). The ultra-fast GC technique coupled with chemometrics allowed to distinguish juice samples containing only 0.5% of impurities. Developed methology is a promising analytical method for the ensurance of quality and authenticity of juices.

1. Introduction

Juice manufacturing is one of the major food industries. Due to the fact that the fruits can rot and their production is seasonal, the production of juices from this kind of raw materials allows us to consume them all year round. Fruit juices and fruit concentrates have become valuable products and intermediates. Many fruit types are used to produce fruit juices, including apples, grapes or peaches, but the most commonly used raw materials are oranges [1]. Food authentication is increasingly important in the food industry, where manufacturers and suppliers can increase their profits by replacing or adding cheaper ingredients to their products [2]. The most common type of juice adulteration is dilution with water, addition of sugar syrup which reduces the total amino acid value, the addition of ingredients that are not naturally present in the juice (e.g., dyes) and of cheaper fruit juices [3]. Orange juices are most often adulterated with addition of mandarin [3, 4], tangerine [5], lemon [6] or grapefruit [7] juices.

Production of orange juice in Poland is based primarily on raw materials imported from Brazil. According to FAO (Food and Agriculture Organization of the

United Nations) statistics, in recent years, the volume of oranges produced in Brazil has decreased by about 15% (data for 2011–2014) [8]. On the other hand, the amount of apples produced in Poland in 2011–2014 has increased by over 20% [9]. The decrease in production of oranges is associated with their higher price, as well as the lesser availability of raw materials. For this reason, orange juice produced in Poland can be adulterated by the addition of apple juice, a cheaper and more easily accessible raw material.

There are many publications that provide information on the chemical, physical and microbiological procedures used to assess the quality of juices. In general, the content of naturally occurring substances in the raw materials is analyzed and the presence of other decomposition products or impurities is detected [10]. The most effective methods are based on the profiling of carbohydrates, phenols, carotenoids, amino acids, or other organic acids using different chromatographic techniques [7]. The use of these procedures is time-consuming. For this reason, new solutions that allow for a rapid assessment of the quality of fruit juices are sought.

The aim of the study was to develop a research methodology for rapid evaluation of the authenticity of orange juices. The developed procedure should be characterized by: no time-consuming sample preparation step, high throughput, repeatability and sensitivity. Methodology allows rapid chemical profiling of raw juices, and in combination with chemometrics can become a powerful tool to evaluate whether a given juice is adulterated.

2. Experimental

2.1 Reagents and chemicals

Fruit juices were obtained at local distribution centres in Gdansk. Samples were NFC (Not From Concentrate) juices, i.e., orange juice, apple juice and mixtures of orange and apple juice (0.5 / 1 / 2 / 3 / 5 / 10 / 20 / 30 / 40 / 50% *v/v* addition of apple juice). A sample of 5 grams of each fruit juice was poured into 20 ml glass vials that were then sealed with a cap with a silicone-PTFE membrane.

2.2 Instrumentation

The electronic nose based on ultrafast gas chromatography (Heracles II, Alpha M.O.S., France) was used for the analysis. Shown in Fig. 1 is a scheme of Heracles II. It is equipped with an injector, sorption trap, two parallel chromatographic columns with different stationary phases and two ultra-sensitive flame ionization detectors (μFIDs). The first column was MTX-5 with a nonpolar stationary phase and the second was MXT-1701 with a medium polar stationary phase. A sample volume of 2.5 ml was introduced into the system using an autosampler (HS 100, Gerstel, Germany). Incubation was conducted at temperature of 40 °C for 2 min.

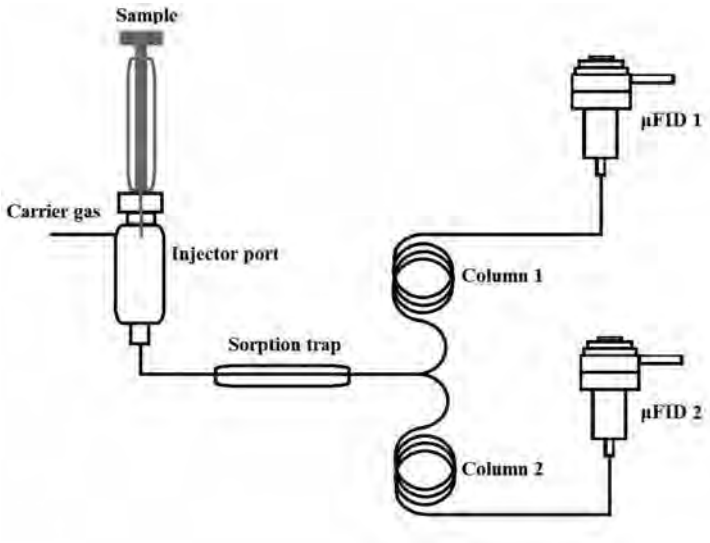

Fig. 1 Scheme of the electronic nose based on ultra-fast gas chromatography.

After incubation, the a portion of the sample's headspace was transferred from the vial to the injector kept at 200 °C. The separation of analytes was performed using the following chromatographic temperature program: initial temperature 40 °C kept for 5 s, then ramped at 4 °C/s to 270 °C and kept for 30 s. Hydrogen with purity of N5.0 was used as a carrier gas. Detector temperature was 270 °C. During the research for each sample 10 repetitions were performed. Data analysis was performed using AlphaSoft v. 12.4 software.

3. Results and discussion

Shown in Fig. 2 (next page) are the radar maps for two groups of fruit juices samples (100% orange juice and mixture of 50% orange juice and 50% apple juice). These radar maps were obtained using the electronic nose based on ultra-fast GC (for two columns: MTX-5 and MTX-1701). The comparison of radar maps showed significant differences in volatile compounds profile of pure orange juice and orange juice with addition of apple juice. Radar maps show that these two groups of samples have comparable intensity of aroma profile. The number of peaks collected for the adulterated juice was larger than the number of peaks registered for pure orange juice. The chromatographic peaks marked on Fig. 2 were only detected in the samples of the juices mixture. These peaks correspond to the chemical compounds characteristic for apple juice. The identification of these compounds is the basis for finding potential authenticity indicators for juices, which would allow to detection of orange juice adulteration.

After the chromatographic analysis, the results were interpreted. The areas of the chromatographic peaks corresponding to the detected chemical compounds

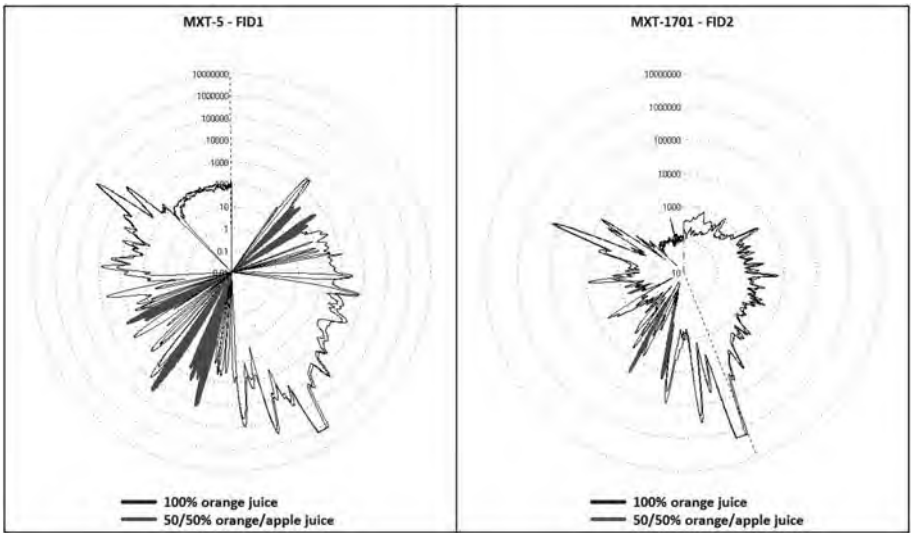

Fig. 2 Radar plot obtained by conducting analysis of 100% orange juice and 50/50% orange/apple juice.

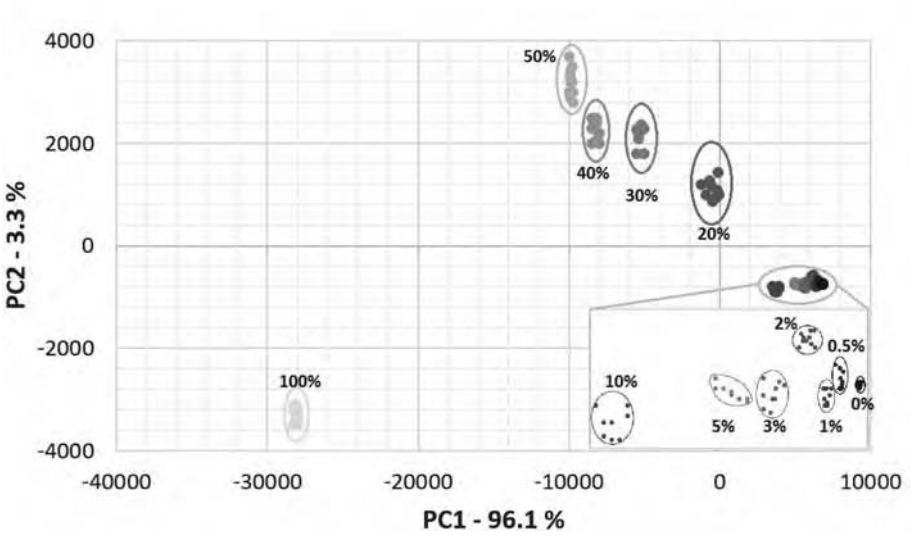

Fig. 3 PCA results for juices samples.

were used as input data for chemometric analysis. All tested samples were plotted in a multi-dimensional space and analyzed on the basis of similarities and differences in the data. During research, Principal Component Analysis (PCA) was used as a chemometric model.

PCA is the most commonly used chemometric technique for analyzing multidimensional datasets. It is used to reduce the number of variables describing a given

dataset and to detect the relation between these variables. This statistical method finds sets of components that are linear. Due to this fact, PCA can be used for classification of samples. Shown in Fig. 3 is the result of the PCA analysis for: orange juice samples (marked as 0% because it contains no apple juice), apple juice samples (marked as 100% because it contains only apple juice), and mixtures of orange and apple juices (indicated by numbers which correspond to the percentage of added apple juice). Based on this graph, it can be observed that all groups of samples could be distinguished. The combination of ultrafast gas chromatography and chemometric method (PCA) allowed to distinguish between samples containing 0.5% by volume of apple juice added. However, it can be seen on Fig. 3, that samples containing from 0.5% to 5% apple juice were lying in small distance from each other. This could make it difficult to distinguish these samples in case when the number of samples will be larger.

4. Conclusions

The use of e-nose based on ultrafast gas chromatography equipped with chemometric method such as PCA is an effective tool which is characterized by a relative short time of a single analysis and no sample preparation stage. Based on the obtained results it can be concluded that it is possible to distinguish between particular orange juice samples based on the added volume of apple juice. The obtained results are the basis for further research. In the future, focus should be placed on the identification of juice quality indicators that will allow to evaluate whether a given juice sample is adulterated. The next step should be to test the developed methodology on samples of fruit nectars and juices from concentrate, because they are more often adulterated.

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Analysis of Volatile Fraction of Hybrid Fruit Pulp Using Proton Transfer Reaction-Time-of-Flight Mass Spectrometry

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Keywords
citrus fruits
food analysis
hybrid fruits
proton transfer reaction–mass spectrometry (PTR)

Abstract
Fruits and vegetables are important ingredients in human diet. Because of this, it is very important to know their exact composition and to control their quality. Based on the aroma of food products, it is possible to pre-evaluate their quality and freshness. The aim of this work was to characterize the volatile fraction of the hybrid fruit "sweetie" using a modern analytical device proton transfer mass spectrometry coupled with time of flight analyser (PTR-TOFMS). Hybrid fruit samples are human-made species that are gaining increasing interest in the food market because of to their attractive features: taste, smell and appearance. It has been demonstrated that applied analytical technique can be used to control the quality of food products, to assess their authenticity and simultaneous do quality analysis.

1. Introduction

Citrus fruits are one of the most commonly consumed species of fruit in the world [1]. For this reason, they are subject of many researches which are focussed on understanding their impact on the human body. Citrus fruits are a good source of many vitamins and minerals necessary for the functioning of the human body. Furthermore, it has been found that many of the chemical substances, found in citrus fruits, have antioxidant properties, which can promote the elimination of cancer cells [2–3]. Sweetie fruit (*C. paradisi × C. grandis*) is a hybrid between pummelo and grapefruit invented in 1962. It is also called green grapefruit because of its characteristic green peel of this fruit [4]. The taste of the fruit resembles the taste of grapefruit but it`s much sweeter. Because of characteristic taste and look sweetie fruit has become very popular in recent years. Based on the aroma of food products (also fruits), it is possible to pre-determine their taste and quality. An analysis of the citrus aroma composition allows it is later reconstitution and use it in many areas of industry, for example: food, pharmaceutical or

chemical industry. In addition, knowing the exact composition of the volatile fraction of the citrus fruits may be the basis for determining the discriminants characteristics for each species of citrus fruits. These discriminants may be useful for future evaluation of the quality and authenticity of selected foods.

Proton transfer reaction–time-of-flight mass spectrometry has been designed, among others, for the determination of volatile organic compounds and the qualitative and quantitative analysis of many other real-time gases. It is a very useful technique with many advantages, for example: there is no sample preparation step required, low detection limit (concentration levels ppt), the duration of the analysis is very short (one measurement takes from a few to several seconds), "green technology" does not require the use of environmentally harmful solvents, determination of analytes without prior calibration using certified reference materials. So far, the device has been used, inter alia, for environmental monitoring, industrial process control and scientific research in many fields such as medicine [5–6].

2. Experimental

2.1 Reagents and chemicals

The fruit was purchased at local markets in Gdańsk, Poland. To prepare the fruit for analysis, each fruit was washed with deionized water. The subject of the study was fruit pulp. Each fruit was peeled to extract the pulp. 5.0±0.1 g of crushed pulp, then 1 ml of deionized water was added into 20 ml glass vials. The vials were closed with a silicone-PTFE membrane caps, then the contents of the vials were gently mixed.

2.2 Instrumentation

Proton transfer mass spectrometry coupled with time of flight analyser (PTR-TOFMS) was used (Ionicon, Austria). The analysis was done in the standard configuration of the used device. The data were collected over a mass range of *m/z* from 12 up to 245. H_3O^+ ions were generated from a pure water vapor (flow of 6 ml). The ionisation conditions were controlled by following parameters: p_{drift} = 2.2 mbar, T_{drift} = 333 K, U_{drift} = 600 V. The volatile fraction was collected by peek tube (110 °C, 0.055 in. diameter) which was linked between the inlet of the PTR-MS and the vials with samples. Analysis of the volatiles was carried out for 15 seconds And the flow rate was 100 ml/min. Analysis of collected data was done using Orange data mining software and chemometric methods (PCA, hierarchical clustering).

3. Results and discussion

The purpose of the research was a characterization of the volatile fraction of the hybrid fruit pulp, sweetie (*C. paradisi × C. grandis*). It was also conducted to

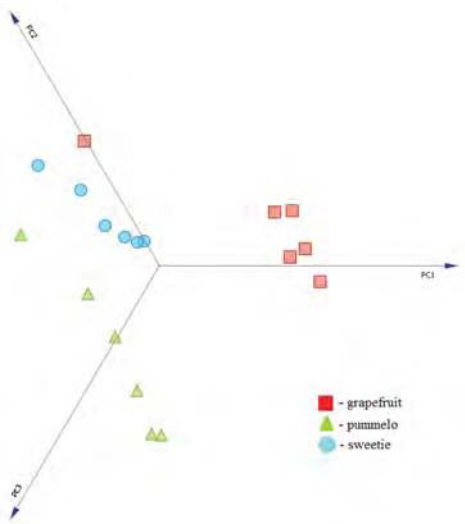

Fig. 1 A graph showing the results of the Principle Component Analysis (PCA) of tested citrus fruits: pummelo, white grapefruit and sweetie using the PTR-TOFMS.

compare and distinguish it with the volatile fractions of the fruit from which this hybrid was formed: pummelo (*C. grandis*) and white grapefruit (*C. paradisi*). The volatile fractions of pulp citrus fruits: pummelo, white grapefruit and a hybrid of those two sweetie were analysed using PTR-TOFMS. The obtained data were analyzed by the Principle Component Analysis (PCA), and the results shown in the graph, in the Fig. 1. Based on these results, it was found that use the PTR-TOFMS device makes it possible to distinguish and classify the examined citrus fruits. Fig. 1 shows points of different shapes, where each shape is characteristic of a given fruit. It is evident that the points are arranged in groups: points are clustered for each shape, which means that the volatile fraction of each fruit significantly differs from the others.

It was also done a cluster analysis. The results of the study were presented in the form of a hierarchical graph in Fig. 2. It can be stated that four different groups were distinguished. The three distinguished groups represent the citrus fruit grade, while the fourth group contains two points, which in fact come from two different citrus fruits. The emergence of the fourth group may affected unavoidable measurement errors, which are influenced by many different factors (e.g., environmental conditions, complicated composition of sample matrix). Based on the cluster analysis, it is possible to determine the degree of similarity of the citrus fruits. Clusters of grapefruit and sweetie are located closer to each other and connected to each other. These are the groups most similar to each other. There was a much smaller similarity of pomelo fruit to the fruits of white grapefruit and sweetie.

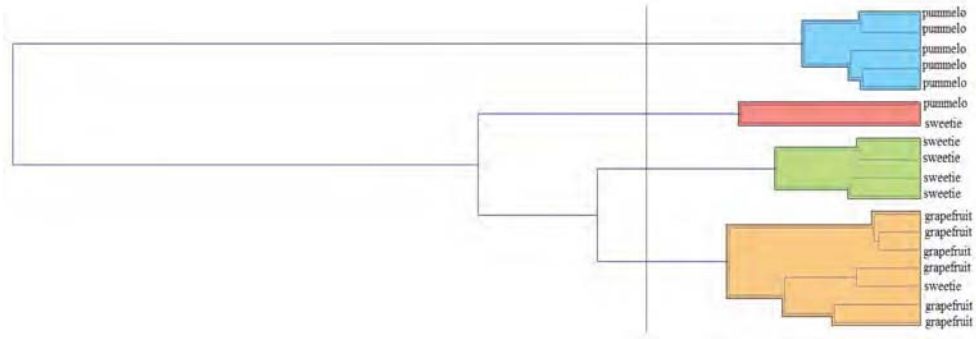

Fig. 2 Graph showing the result of cluster analysis of citrus fruits pulp: pummelo, white grapefruit and sweetie using PTR-TOFMS.

Table 1
Selected chemical compounds identified on the basis of mass spectra obtained in the volatile fraction of sweetie fruit pulp using PTR-TOFMS.

Chemical compound	Aroma of chemical compound
D-limonene	citrus aroma
myrcene	mossy aroma
α-pinene	pine tree aroma
hexanol	green aroma
ethyl acetate	fruity aroma
ethyl butanoate	fruity aroma
acetic acid	sour aroma
pentanol	fruity aroma

In the Table 1 is shown selected chemical compounds identified from the mass spectrum obtained using PTR-TOFMS. The same chemicals were detected in the grapefruit's and pummelo's volatile fractions [7–8]. These results confirm the results of studies carried out on citrus fruit by other scientists. Chemical compounds listed in Table 1 are characteristic for citrus fruits. Their presence in the fruity volatile fraction of citrus fruits causes a known aroma of these fruits.

4. Conclusions

Results of PTR-TOFMS analysis allowed classifying the samples of pulp of citrus fruits. It also allowed to determine the degree of similarity of the volatile fractions of fruit's pulp. Based on the results of PCA analysis, it has been shown that PTR-TOFMS device to classify species and clearly distinguish the volatile fraction of the pulp tested fruits is an effective technique for this purpose. Based on the results obtained from the cluster analysis, it has also been shown that it is possible to distinguish citrus fruit. In addition, it was found that the aroma of white

grapefruit and sweetie is very similar. Much easier it is to distinguish aroma of pummelo and sweetie than grapefruit and sweetie, because of much less similarity aroma of pummelo and sweetie. Short time of analysis and simple sample pre-paration makes this instrument a useful tool for monitoring the quality of samples on-line, for example: samples of food. Despite, the complex matrix of the examined samples and the background pollution, it was possible to perform an effective assessment of the similarity and differentiation of the examined fruit pulp.

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Analysis of Odor Interactions in Ternary Gas Mixtures Using Electronic Nose

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Keywords
electronic nose
model
odor intensity
odor interactions
sensory analysis

Abstract
In recent years there has been a noticeable increase in interest in the use of electronic noses in analytical chemistry. These devices perform a holistic analysis of the gas mixtures composition, without the separation and identification of its individual components. For this reason e-noses are increasingly replacing the conventional olfactometers, due to the significantly shorter analysis time and possible automation. In the work the authors attempt to analyze odor interactions in ternary gas mixtures using the electronic nose. Obtained results were compared with sensory analysis results and theoretical values (calculated using perceptual Zwaardemaker model). As a result of the studies, it was found that it is possible to use e-noses instead of olfactometers because the areas of odor interactions determined by the instrumental method overlap to the areas indicated by the sensory panel.

1. Introduction

Nowadays, more and more frequent problem of population living in big cities is the occurrence of odor nuisance. Although the source of these nuisances is different (e.g., wastewater treatment plants, municipal landfills), their common feature is that they are a complex mixture of odorants with different odor thresholds [1, 2]. Therefore, an important problem is measurement and monitoring of air quality in terms of odor nuisance. At present, odor concentrations are determined by the standard olfactometry method EN 13725:2003 *Air Quality – Determination of Odor Concentration by Dynamic Olfactometry*. However, from a practical point of view, it would be most valuable to directly link the odor intensity with the results of analytical air monitoring. This would allow the on-line odor monitoring using electronic noses, which due to the shorter time of analysis and the possible automation are increasingly replacing traditional measurements based on olfactometry techniques. One of the advantages of olfactometry is taking into account

Table 1
Perceptual model examples (*I* is odor intensity; *k*, α_{AB}, α_U are empirical constants specific to a given pair A + B).

Model	Authors
$I_{AB} = \sqrt{I_A^2 + I_B^2 + 2I_A I_B \cos\alpha_{AB}}$	Zwaardemaker
$I_{AB} = k(I_A + I_B)$	Berglund, Lindvall
$I_{AB} = \sqrt{I_A^2 + I_B^2}$	Patte, Laffort
$I_{AB} = I_A + I_B + 2\sqrt{I_A I_B}\cos\alpha_U$	Patte, Laffort

the interactions (synergy, inhibition, masking) between odor mixture components. The aim of this research is to demonstrate that these these interactions can be also described using the electronic nose technique.

One of the most commonly determined feature of the smell is the odor intensity. It is defined as the perceived strength of odor sensation that will be triggered by a specific stimulus. In the case of individual substances, the relationship between the odor intensity and the concentration can be described by Weber-Fechner (1) or Stevens's power (2) law

$$I = k_{WF} \log \frac{c}{c_{OT}} \tag{1}$$

$$I = k_S \times c^n \tag{2}$$

where *I* is odor intensity, k_{WF}, k_S, *n* are individual empirical constants determined for each substance, *c* is substance concentration, and c_{OT} is odor threshold.

In the case of multi-component mixtures, due to the presence of odor interactions, the odor intensity is not an additive value. Several mathematical models have been developed to predict the quantitative interactions in binary mixtures on the basis of perceived odor intensities of the unmixed components. The examples of perceptual models are shown in Table 1. The model proposed by Zwaardemaker (vector addition) can be used to theoretically determine the odor intensity of a ternary mixture

$$I_{ABC}^2 = I_a^2 + I_b^2 + I_c^2 + 2\left(I_a I_b \cos\alpha_{ab} + I_b I_c \cos\alpha_{bc} + I_a I_c \cos\alpha_{ac}\right) \tag{3}$$

The interaction coefficient (α) in the Zwaardemaker equation (3) is approximately constant for the given pair of components of the mixture. The literature shows that its value is generally in the range of 102–115 ° [3].

Table 2
Components of the electronic nose system.

System component	Description
Sampling system	It eliminates all undesirable factors that can affect the sensor response and provide stable and reproducible measurement conditions (temperature, humidity, gas flow velocity).
Detection system	A set of sensors located in the measurement chamber. The most commonly used type of sensors are commercially available sensors for detection of volatile organic compounds, e.g., Metal Oxide Sensors (MOS) [4]. They exhibit different selectivity and sensitivity, but as a whole, produce a characteristic chemical image of the gas mixture ("fingerprint").
Data collection system	It is responsible for signal processing.
Pattern recognition system	It assigns the received set of signals to one of the pattern classes.

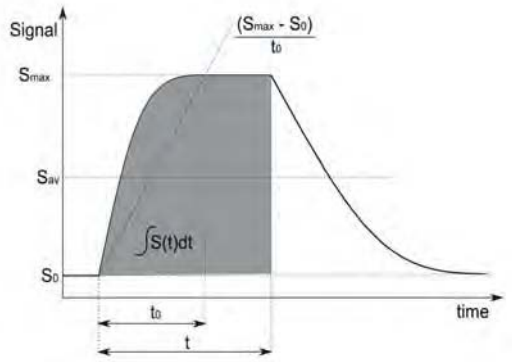

Fig. 1 Signal parameters used for data analysis: S_{max} is maximum signal value, S_{av} is average signal value, $\int S(t)\mathrm{d}t$ is integral of sensor signal, $(S_{max} - S_0)/t_0$ is difference quotient of signal.

In recent years there has been a noticeable increase in interest in the use of artificial senses in analytical chemistry. Particular attention is paid to electronic noses; devices that are supposed to imitate the human sense of smell. The electronic nose system consists of four main components, which are described in Table 2.

An example of an e-nose sensor response as a function of the analysis time together with the signal parameters used for data analysis is shown in Figure 1. The most commonly used parameter is the maximum signal value in the specified time interval (S_{max}).

Electronic noses, as well as olfactometric techniques, perform a holistic analysis of the gas mixture composition. Consequently, their use in the study of odor interactions seems to be reasonable and expedient, as opposed to chromatographic methods which only provide information on the individual components concentrations. Previous studies have shown that it is possible to determine the linear relationship between odor intensity and e-nose sensor signal. Using this

Table 3
Odor properties of used chemical compounds.

Chemical compound	Odor detection threshold[a] / ppm	Odor type
acetone	42	pungent, characteristic
α-pinene	0.018	pine-like
triethylamine	0.0054	strong fishy odor reminiscent of ammonia

[a] Ref. [6].

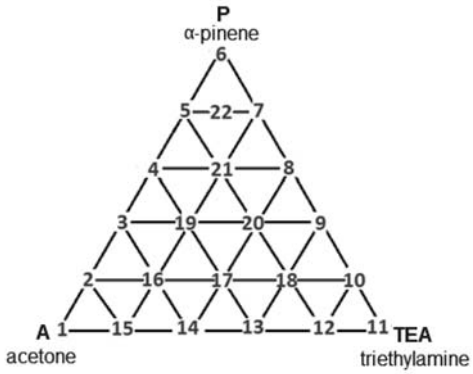

Fig. 2 A ternary plot illustrating the composition of the tested mixtures.

relationship, a linear model combining the odor intensity with the values of the signal was proposed

$$I = a_0 + a_1 \log S_{\max,1} + a_2 \log S_{\max,2} + \ldots + a_n \log S_{\max,n} \quad (4)$$

The proposed model was characterized by a good determination coefficient in determining the odor intensity of perfumes samples [5]. For this reason, it has been used in presented research, showing the possibility of analyzing odor interactions in ternary gas mixtures using electronic nose. The results were compared to the values obtained from the Zwaardemaker perceptual model and sensory analysis.

2. Experimental

2.1 Reagents and chemicals

Aqueous solutions of acetone, α-pinene and triethylamine were prepared to perform sensory tests and to develop the model (4). The chemicals (Sigma-Aldrich) were of analytical reagent grade. Their properties are shown in Table 3. Twenty two solutions were prepared for each analysis. Their composition corresponds to the position of the points in the triangle representing the ternary system (Fig. 2).

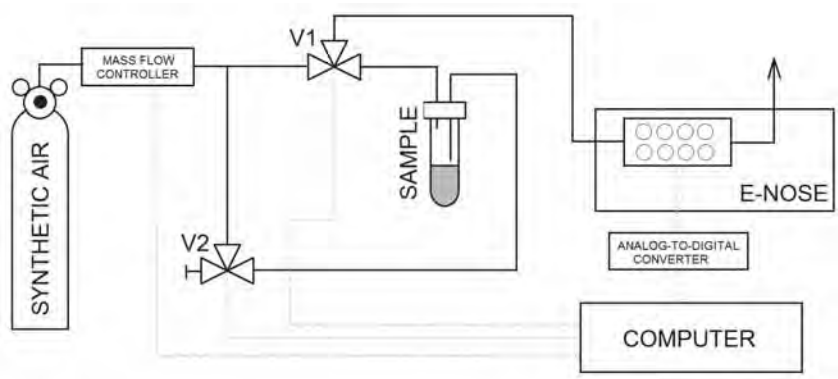

Fig. 3 Electronic-nose measurement system.

The primary samples of individual substances (1, 6, and 11) were prepared in such a way that they had an odor intensity equal to one. These concentrations were respectively: 600 ppm *v/v* acetone, 0.1 ppm *v/v* α-pinene and, 1.5 ppm *v/v* triethylamine. Further samples were prepared from primary solutions in the proportions determined by their position in the ternary plot.

2.2 Sensory analysis

Sensory evaluation of odor intensity was carried out by four persons, selected according to the procedure described in [7]. Each member of the panel was responsible for assigning the appropriate odor intensity value to a given sample using a 7-step scale described in German Standard VDI 3940.

2.3 Electronic nose analysis

The prepared samples were analyzed using a constructed electronic nose prototype. The device was equipped with eight metal oxide sensors manufactured by Figaro Engineering: TGS2104, TGS2106, TGS2180, TGS2600, TGS2602, TGS2201A, TGS2201B, and TGS2611. The scheme of the measurement system is shown in Fig. 3.

Purified air flow through a system at a constant flow rate of 300 $cm^3 min^{-1}$ was controlled by a mass flow controller. The headspace analysis of prepared samples was conducted. By changing the position of the solenoid valves, the air flowed through the sample, which then passed to the measurement chamber. The electronic nose worked in the stop-flow mode: the sample flow time was 25 seconds and the stop time of the mixture in the sensors chamber 10 seconds. After this time the purified air was returned to the measurement chamber for regeneration of the sensors. Signals from the sensors were recorded using an ADC converter (Simex SIAi-8) and saved on the computer. Data analysis and other calculations were performed using RStudio Desktop (v. 1.0.143) software.

Table 4
Calculated model parameters and their statistical significance.

Coefficient	Coefficient value	Statistical significance
a_0	–11.41	yes
a_1	–4.58	yes
a_2	1.61	no
a_3	–0.21	no
a_4	14.64	yes
a_5	–2.31	no
a_6	0.28	no
a_7	–0.2	no
a_8	2.32	no

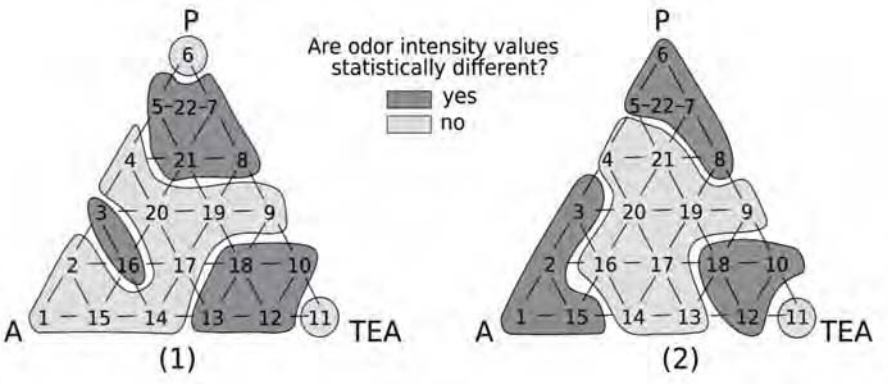

Fig. 4 Ternary plots of gas mixtures odor intensity statistical differences: (1) determined by the sensory analysis, (2) modeled using the e-nose, compared to the values calculated using the Zwaardemaker model. Abbreviations: (P) α-pinene, (A) acetone, (TEA) triethylamine.

3. Results and discussion

Based on the sensory analysis of 22 samples, a linear model (4) has been developed. Table 4 shows the calculated model parameters (R^2 = 0.74). Their statistical significance was evaluated using *t*-statistics for significance level α = 0.05 and the number degrees of freedom equal to 35. By examining the model coefficients presented in Table 4, it was found that only S_1 (TGS2104) and S_4 (TGS2600) sensors introduce analytically useful information to the model. After rejecting statistically non-significant sensors signals from the model, the regression coefficients were recalculated. The model (5) with a determination coefficient of 0.85 was obtained

$$I = -9.35 - 5.60 \log S_1 + 14.73 \log S_4 \qquad (5)$$

The developed model was used to determine the odor intensity of the samples analyzed by the electronic nose. The statistical differences between the values pairs (e-nose – theoretical model and sensory analysis – theoretical model) are shown in Fig. 4.

No statistically significant difference was observed for most samples, which indicates that there were no odor interactions between mixture components. In the sensory analysis results, odor intensity strengthening was observed for the high content of α-pinene and triethylamine in the mixture (in the corners of the triangle). A similar relationship was noted for the results obtained using the electronic nose. The differences only occur in the area of high acetone content. In this case e-nose correctly defined only one increase of odor intensity (sample 3).

4. Conclusions

As a result of the studies, it was found that the electronic nose prototype along with the developed linear mathematical model can be successfully used to estimate the odor intensity of α-pinene, acetone and triethylamine mixtures. The proposed model was characterized by a high determination coefficient $R^2 = 0.85$. It has also been shown that both: the sensory panel and the prototype of the electronic nose indicate the intensification of the odor intensity of some samples. The research has shown that the use of e-noses instead of olfactometers is possible because the areas of odor interactions determined by the instrumental method overlap in 72% to the areas indicated by the sensory panel.

Acknowledgments

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Development of a Tube Nebulizer for Microflow Atmospheric Pressure Chemical Ionization Mass Spectrometry

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Keywords
mass spectrometry
micro-APCI
tube nebulizer

Abstract
A novel nebulizer for microflow atmospheric pressure chemical ionization mass spectrometry was developed. It consisted of a tube with analyte-transporting fused silica capillary inside. The terminal part of the tube was resistively heated using a coiled resistance wire. Optimal nebulizer parameters and construction setup were investigated to fully demonstrate its versatility. Corundum appeared to be a more suitable material than quartz for fabrication of the tube nebulizer. The lowest analyte flow rate investigated was 1.0 µl/min with an excellent signal stability reached.

1. Introduction

Nowadays, atmospheric pressure chemical ionization (APCI) is an important tool for organic mass spectrometry [1]. It is not used as much as electrospray ionization (ESI) but it is of significant importance for the analysis of certain classes of compounds (e.g., lipids) [2]. In addition, non-polar solvents cannot be used in direct ESI detection [3], while in APCI they can be used without any difficulties in normal-phase HPLC [4].

The flow rates used for a conventional APCI source are relatively high, typically 50–1000 µl/min [5]. Nevertheless, one of the current trends in analytical chemistry is the miniaturization of analytical instruments [6]. Miniaturized sources can be coupled with low-flow liquid chromatography or microfluidic systems. APCI source miniaturization could decrease the quantity of sample used for analysis, and, as it is seen in nano-ESI source, improve the sensitivity [5]. Several works have been done in this respect, especially with microchip nebulizers [5, 7]. Unfortunately, microchips have fixed constructions, so their parameters cannot be easily optimized. In this work, we demonstrate a micro-APCI source based on a tube nebulizer for flow rates ranging from 1 to 10 µl/min. Its simple design makes it possible to optimize the working setup easily.

2. Experimental

2.1 Materials

Acridine (97%), reserpine, acetonitrile (for MS) and toluene (HPLC grade) were obtained from Sigma-Aldrich (USA), and cholesterol standard was from Lachema (Czech Republic). Solutions of acridine and reserpine were prepared in acetonitrile (50 μmol/l) and cholesterol was dissolved in toluene (260 μmol/l).

2.2 Instrumentation

An ion trap mass spectrometer LCQ Fleet (Thermo Fisher Scientific) was used. For all measurements, the parameters were set as follows: sheath gas 20 a.u., capillary temperature 300 °C, capillary voltage 25.7 V, tube lens 120 V. Auxiliary gas was not used in any measurement. The spectra were recorded for 1 minute. An exception was the stability experiments where the data were collected for 9 minutes.

2.3 Micro-APCI source setup

The nebulizer for micro-APCI source was realized as a heated tube with a polyimide-coated fused silica capillary positioned inside. The analyte solution was introduced from a syringe pump via a polyetheretherketone (PEEK) capillary to the fused silica capillary, which were joined together using a PEEK union. The fused silica capillary was inserted into and passed through a low-pressure PEEK cross. The PEEK cross was attached to a plastic board which was mounted on a custom-made *x-y-z* movable table. The cross served as a junction for the fused silica capillary and nebulizing gas flow which were introduced perpendicularly to each other. Nebulizing gas (nitrogen) was delivered from the mass spectrometer through a GFC mass flow controller (Aalborg) which provided a precise regulation of nitrogen flow rate. The cross orifice opposite to the gas entrance was plugged. The fused silica capillary protruded into the nebulizing tube which was fixed to the cross using a 1/8" nut. Two nebulizing tubes were tested, one made of quartz (outer diameter: 2.8 mm, inner diameter: 1.3 mm, length: 118 mm) and the other one made of corundum (outer diameter: 2.1 mm, inner diameter: 1.1 mm, length: 92 mm). A metal wire spring was used to maintain the fused silica capillary in the center of the nebulizing tube. The last 3 cm of the nebulizing tubes were heated with a KanthalTM resistance wire which was tightly coiled around them. Each of the tubes had its own wire due to their different outer diameters. The number of wire threads was 27 for the quartz tube and 41 for the corundum tube. Electric power applied to the wires was controlled by an external voltage source Manson HCS-3202-000G (Manson Engineering Industrial, Hong Kong). Two positions of the capillary ending were tested, first when the fused silica capillary penetrated into the heating zone and second when it ended right before it. The first setup was

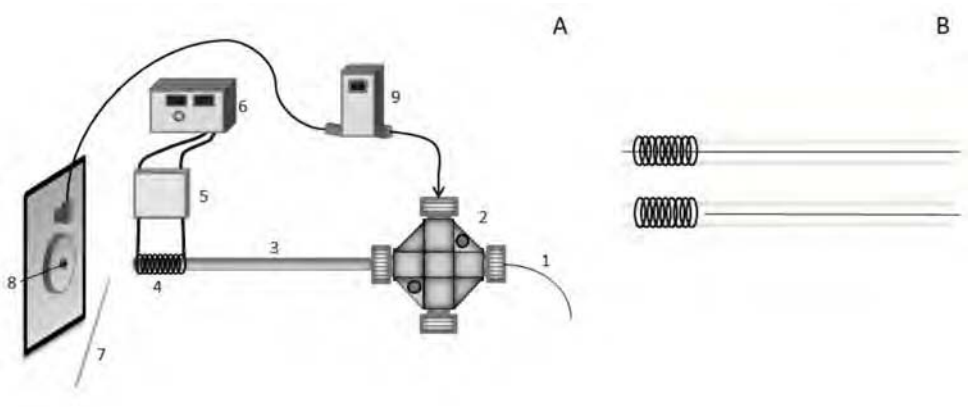

Fig. 1 (a) Schematic view of a micro-APCI source with a tube nebulizer and (b) two different positions of fused silica capillary ending. Fused silica capillary (1), low-pressure PEEK cross (2), tube (3), resistance wire (4), ceramic terminal block (5), power supply (6), APCI needle (7), MS inlet (8), mass flow controller (9).

used for the tube comparison and the second one for signal stability tests with corundum tube. In both cases, polyimide was intentionally burned off from the terminal part (4 cm) of the fused silica capillary. Otherwise, undesirable MS signal would be detected. The solution coming from fused silica capillary was evaporated in the heating zone, and after that, it was led co-axially to MS inlet by nebulizing gas. The analytes were ionized by the corona discharge generated on the APCI needle, which was mounted on a custom-made holder. The discharge current applied to the needle was set to 1.0 μA. The distance between the tube and MS inlet was 1 cm, and the needle was placed slightly off-axis, 0.3 cm from MS inlet (Fig. 1).

3. Results and Discussion

3.1 APCI needle position

The position of APCI needle in the ion source appeared to be very important. When the tip of the needle was too close to the MS inlet, undesirable and potentially harmful electric sparks were observed. The same was observed when the needle was placed in a short distance from the resistance wire. An optimal position was found to be 0.3 cm from the MS inlet and 0.7 cm from the resistance wire when the ionization was most efficient and no sparks were observed. The needle tip was localized slightly off-axis. When it was directly in the way of the ions, the positive charge of the needle tip redirected part of the cations out of the MS inlet which led to lower signal intensities.

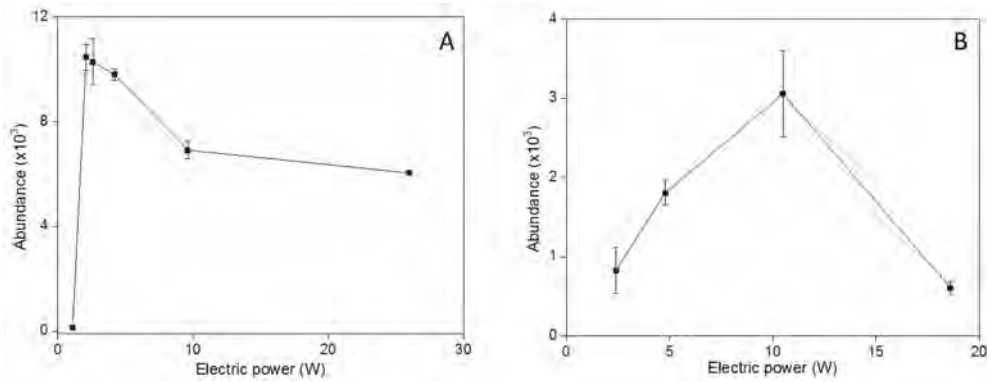

Fig. 2 Absolute signals of acridine as a function of electric power, which was applied to the resistance wire of the (a) quartz tube and (b) corundum tube.

3.2 Resistance heating

High temperature served for evaporation of the solvent coming from the fused silica capillary. When low temperatures were used, poor or no signal of the analyte was detected due to the weak solvent evaporation. On the other hand, very high temperatures caused decomposition of the analyte and its deposition within the fused silica capillary, leading to a quick clogging. In addition, high temperatures tend to weaken and ultimately damage the resistance wire. For these reasons, an optimum heating power had to be established. The best heating power was found to be 2.1 W for the quartz tube and 10.5 W for the corundum tube (Fig. 2). This is in contrast with expectations because the corundum tube had a smaller diameter, and corundum has a higher heat conductivity than quartz [8].

3.3 Nebulizing gas flow

Signal intensity. Just like in conventional APCI, nebulizing gas flow is a crucial parameter for both intensity and stability of analyte signal. Nebulizing gas transports analyte from the end of fused silica capillary to the MS inlet. While low gas flow rates make the analyte transport inefficient, high gas flow rates direct most of the analyte ions outside the MS inlet. Therefore, the best gas flow for both tubes was investigated. The biggest signal intensity for quartz and corundum tube was achieved with nebulizing gas flow 220 ml/min and 100 ml/min, respectively. This difference is likely caused by the fact that the inner diameter of the corundum tube is smaller than the inner diameter of the quartz tube (Fig. 3a and 3b; next page).

Signal stability. When the fused silica capillary was penetrating the heating zone, the solvent was evaporating inside the capillary and bubbles of gas were created. These bubbles caused signal instability. Therefore, the fused silica capillary was placed before the heating zone. However, only the corundum tube

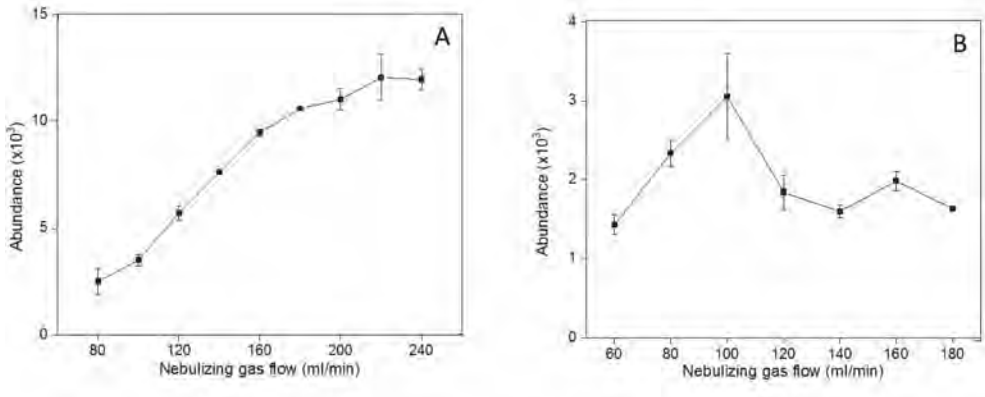

Fig. 3 Absolute signals of acridine as a function of nebulizing gas flow rate for (a) quartz tube and (b) corundum tube.

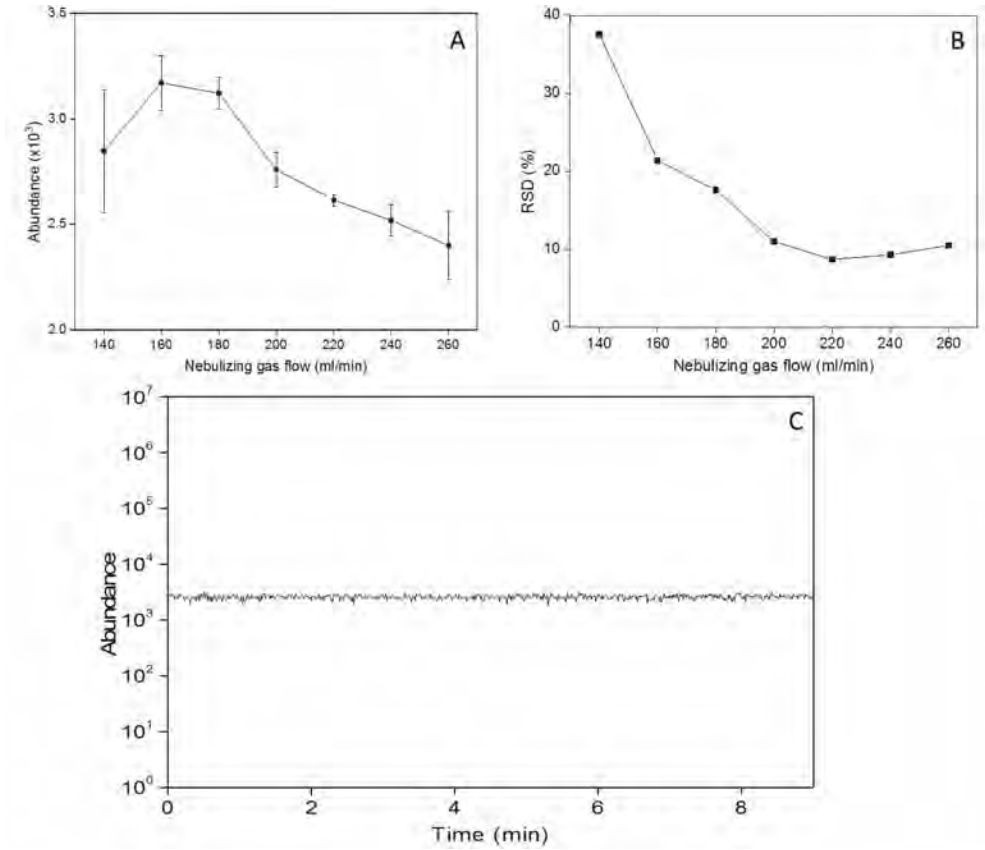

Fig. 4 (a) Absolute signal intensity of acridine as a function of nebulizing gas flow for analyte flow rate 1.0 µl/min, (b) corresponding *RSD* values from the same measurement, (c) stability of absolute acridine signal for gas flow rate 220 ml/min.

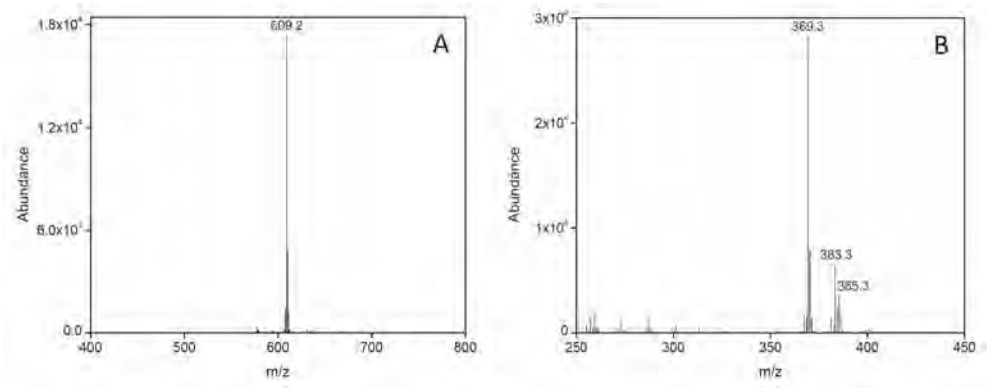

Fig. 5 Mass spectra of (a) reserpine, and (b) cholesterol obtained with micro-APCI source with tube nebulizer.

showed improvement of signal stability. Because of this, the quartz tube was not used for further investigation.

For the new setup mentioned above, analyte flow rate of 1.0 µl/min was used. The highest signal intensity was reached for gas flow 160 ml/min instead of 100 ml/min, which was optimal in the previous experiment (Fig. 4a). This change was likely caused by prolonging the distance between the fused silica capillary and the MS inlet. The lowest relative standard deviation (*RSD*) was reached when nebulizing gas flow 220 ml/min was used (Fig. 4b and 4c). Obviously, the highest signal intensity and the lowest *RSD* have not been obtained at the same gas flow rate (Fig. 4a and 4b). Therefore, a compromise setting of 180 ml/min was chosen.

3.4 Detection of different analytes

Apart from acridine, which was used in all previous measurements, other testing compounds were successfully detected as well. These include reserpine (50 µmol/l in acetonitrile) and cholesterol (260 µmol/l in toluene). As in the case of acridine, reserpine provided mostly $[M + H]^+$ ions (Fig 5a). In the mass spectrum of cholesterol, dehydrated protonated molecules $[M - H_2O + H]^+$ were abundantly formed (Fig. 5b), just like it was observed in commercial high-flow rate APCI source [9]. The ions $m/z = 383$ and $m/z = 385$ corresponded to $[M - 3H]^+$ and $[M - H]^+$, respectively [10].

4. Conclusions

This contribution presents a novel nebulizer for micro-APCI. Acridine and reserpine were successfully detected at the concentration of 50 µmol/l, while the concentration of 260 µmol/l was used for cholesterol detection. Two tubes of different sizes and different materials (quartz and corundum) were tested. After the optimal parameters for both tubes were established, the higher signal

intensity was achieved with quartz tube but signal stability was unsatisfactory for both of them. In addition, clogging of the end of the fused silica capillary became a major inconvenience. This was rapidly improved by removing the capillary from heating zone and setting its end right before it. As a result, an increase of signal intensity and tremendous improvement of its stability was observed when corundum tube was used. This allowed detection of analytes with flow rate as low as 1.0 µl/min. Overall, the corundum tube was proved to have more advantages than the quartz tube. A new design of the nebulizer is being constructed, which will add some new possibilities to the detection process (e.g., switching between ionization in ambient and high temperature).

Acknowledgments

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Electrochemical Sensor Based on Boron Doped Diamond Electrode for Determination of Phenolic Compounds

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Keywords
amperometric detection
boron doped diamond
HPLC
phenolic compounds

Abstract
The electrochemical behaviour of selected phenolic compounds was investigated at boron doped diamond electrode. Anodic activation has to be applied before each scan to ensure repeatable voltammetric response at boron doped diamond electrodes with metallic-type conductivity or semicondutive properties. The isomers of cresols exhibited similar oxidation potentials using differential pulse voltammetry in Britton-Robinson buffer pH = 2.0. Boron doped diamond electrode also succeeded as amperometric sensor in HPLC-ED setup for separation and detection of a mixture of phenolic compounds consisting of *o*-, *m*-, and *p*-cresol, 4-chlorophenol, 4-chlorocresol, hydroquinone, and phenol with problematic separation of *p*- and *m*-cresol on C18 reversed phase column.

1. Introduction

Cresols are aromatic phenols classified by the US-EPA as persistent and toxic chemicals, showing chronic effects at 12 mg L^{-1} estimated by quantitative structure–activity relationship [1, 2]. They are used during pesticides production, polymer production, and in other industrial spheres and thus consequently they may pollute the environment. There are three isomers of cresol exhibiting similar chemical and physical properties, thus it is problematic to determine all these isomers in the mixture and separation step before their detection is usually a necessity. Electrochemical oxidation is one of the common approaches despite the fact that their oxidation is a complicated process involving formation of phenoxy-type radicals in the first one-electron step. Their consecutive reactions are responsible for electrode surface fouling [3].

Boron doped diamond (BDD) electrodes have succeeded as sensors for batch voltammetric determination of a number of phenolic compounds including benzophenone-3 [4], and 4-chloro-3-methylphenol [5]. They are advantageous

for that purpose due to their wide potential window in anodic region and the possibility of in-situ electrochemical activation applying highly positive potential in the region of water decomposition. Further, they can be used as amperometric detectors in flow systems [6, 7].

This contribution aims at utilization of BDD electrodes for batch voltammetric determination of cresols and their determination in the mixture of phenolic pollutants using HPLC with electrochemical detection (HPLC-ED).

2. Experimental

2.1 Reagents and chemicals

Standard solution of cresols (Sigma Aldrich, ≤95%; $c = 1\times10^{-2}$ mol L^{-1}) and other phenols (Sigma Aldrich, ≤95%; $c = 1\times10^{-2}$ mol L^{-1}) were prepared in deionised water. As a supporting electrolyte the Britton-Robinson buffer (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) was used. Acetonitrile (gradient grade) was obtained from Merck (Czech Republic).

2.2 Instrumentation

All voltammetric measurements were carried out by electrochemical analyser AUTOLAB PGSTAT 101 (Metrohm Autolab, The Netherlands) with NOVA (1.11 version) software using three electrode arrangement. The BDD electrodes deposited on silicon discs prepared by using microwave plasma assisted chemical vapour deposition procedure at B/C ratio 500 ppm, 1000 ppm, 4000 ppm, 8000 ppm (Institute of Physics of AS CR, Czech Republic, details in [8]) were used as working electrodes (geometric area 5.72 mm^2). Argentochloride electrode (3.0 mol L^{-1} KCl) was used as reference and platinum wire as auxiliary electrode (both Elektrochemické detektory, Czech Republic). Modulation amplitude of 100 mV, modulation time of 50 ms and interval time of 200 ms was used for differential pulse voltammetry (DPV) for the electrochemical study of cresols. The BDD electrode surface was anodically pre-treated in 0.5 mol L^{-1} sulfuric acid by applying potential +2.4 V for 10 min at the beginning of each working day and activated 1 min in-situ at the same potential before each individual measurement. HPLC instrument Merck HITACHI consisting of D-7000 interface, D-7000 auto-sampler, L-7100 pump, L-7400 UV detector, and BDD electrode (8000 ppm) in wall-jet arrangement as amperometric detector was used in separation and detection of selected model phenols mixture. The separation LiChroCART RP-18 column (125×4 mm, 5 μm) and isocratic conditions of acetonitrile/ Britton-Robinson buffer (pH = 2.0) 40/60 (*v/v*) with flow rate of 1.0 mL min^{-1} was used (injection volume 20 μl). The amperometric detection potential was set at +1.5 V (optimised value).

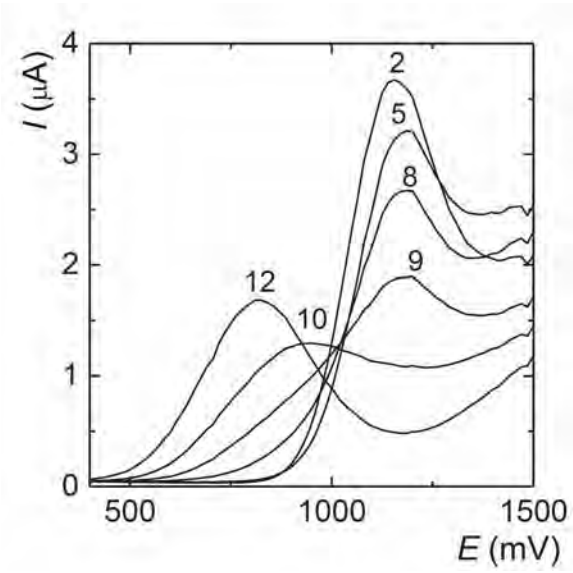

Fig. 1 DP voltammograms of *m*-cresol (1×10^{-4} mol L^{-1}) measured at boron doped diamond electrode in different pH = (2.0–12.0) of Britton-Robinson buffer indicated by the curves.

3. Results and discussion

3.1 Optimization of detection conditions

The pH of supporting electrolyte has large influence on oxidation of phenolic compounds due to their acidic character and possible involvement of protons in the mechanism of oxidation. Acidic pH is frequently used for batch voltammetric determination of phenolic compounds including cresol derivatives [5] and was favourable also for tested cresols. The oxidation potential of *o*-cresol and *p*-cresol was around +1.0 V and potential of *m*-cresol was around +1.1 V in Britton-Robinson buffer pH = 2.0 resulting in well-shaped peaks obtained by DPV, as demonstrated for *m*-cresol in Fig. 1. From the oxidation potentials values it is obvious that for determination of the mixture of isomers it is necessary to use the separation step before electrochemical detection.

Further, the influence of boron content in BDD electrode on DP voltammetric response of cresols was investigated. BDD electrodes with metallic type of conductivity (B/C ratio 8000 ppm and 4000 ppm) and semiconductive electrodes (500 ppm and 1000 ppm) exhibited well-shaped oxidation peaks with decreasing potential with increasing boron content and slight increase of the peak height for the 8000 ppm electrode (for *o*-cresol depicted at Fig. 2). This is indicative of easier oxidation and increased electron transfer kinetics for the metallic-type BDD films.

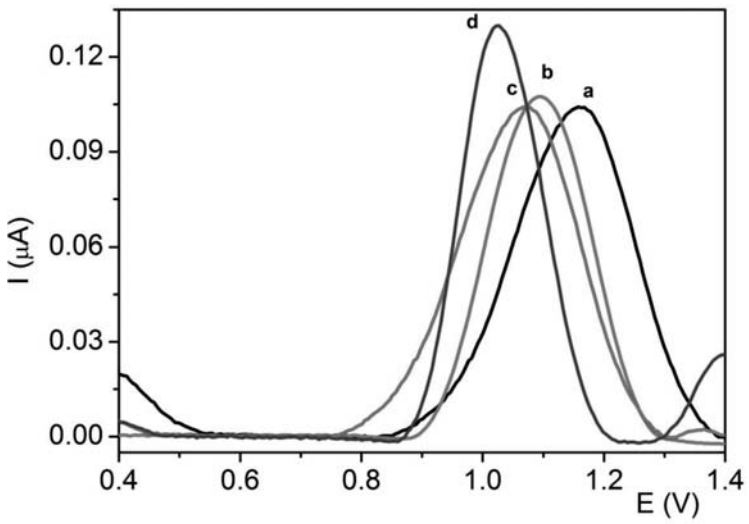

Fig. 2 DP voltammograms of 50 μmol L^{-1} *o*-cresol in Britton-Robinson buffer pH = 2.0 registered at BDD electrodes deposited at B/C ratio: (a) 500, (b) 1000, (c) 4000, (d) 8000 ppm.

3.2 HPLC with amperometric detection

The 8000 ppm electrode was further used for amperometric detection in wall-jet arrangement in HPLC-ED setup for separation and detection of a mixture of phenolic compounds (*o*-, *m*-, and *p*-cresol, 4-chlorophenol, 4-chlorocresol, hydroquinone, and phenol). The detection potential was optimised in the range from +0.5 V to +2.0 V and +1.5 V was chosen as optimal for the highest signal/background current ratio for most of the tested compounds. No activation had to be applied between individual injections, the electrode surface was sufficiently cleaned by the flow of the mobile phase. The separation succeeded in 7 min with absent resolution for *p*- and *m*- cresol. Their problematic separation was reported also in other studies. The other phenolic compounds were separated with sufficient resolution.

4. Conclusions

Cresols are oxidisable at boron doped diamond electrode within its potential window and in-situ anodic activation between individual scans ensuring repeatable voltammetric signals. Due to the similar structure of *o*-, *m*-, and *p*-cresols their oxidation potentials are very close. BDD electrode also succeeded as amperometric sensor in HPLC-ED setup for separation and detection of a mixture of phenolic compounds. The problematic separation of *p*- and *m*- cresol in HPLC with C18 reversed phase column is a challenge for search on more appropriate separation system.

Acknowledgments

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Determination of Inorganic Arsenic in Samples of Marine Origin

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Keywords
hydride generation
inorganic arsenic
speciation analysis

Abstract
A fast screening method for determination of inorganic arsenic in marine samples was developed. This method is based on selective hydride generation and detection by inductively coupled plasma mass spectrometry. Using a high concentration of hydrochloric acid together with hydrogen peroxide for hydride generation leads to selective conversion of inorganic arsenic species into arsane. The accuracy of this method was verified by comparative analyse by high performance liquid chromatography coupled with hydride generation and atomic fluorescence detection.

1. Introduction

The toxicity of arsenic strongly depends on its chemical form. The toxicity of arsenic species varies from the most toxic inorganic species, arsenite (iAs^{III}) and arsenate (iAs^{V}), to much less toxic methylated species, methylarsonate (MAs^{V}), dimethylarsinate ($DMAs^{V}$), and trimethylarsine oxide ($TMAs^{V}O$), to non-toxic species like arsenobetaine and other complex organic species [1]. The determination of inorganic arsenic species in food is an important task, because food is the main contributor to human inorganic arsenic species intake (excluding drinking contaminated water) [2]. The most common method of arsenic speciation analysis is nowadays high performance liquid chromatography (HPLC) hyphenated with inductively coupled plasma mass spectrometry (ICP-MS) or with hydride generation (HG) and atomic fluorescence detection (AFS) [3]. There is an increasing interest in robust and reliable methods to determine the inorganic arsenic species concentration in a range of food because the EU legislation on inorganic arsenic species content in food items might become a reality in the near future [2, 4].

Therefore, the development of simple and fast analytical methods is still necessary with a focus on screening of large number of samples [4]. Recently, a method of selective hydride generation-inductively coupled plasma mass spec-

trometry (HG-ICP-MS) for the fast determination of inorganic arsenic species in rice samples was published [5] and successfully validated for rice samples [6]. The method uses high concentrations of hydrochloric acid (5M) and sodium borohydride for the selective generation of arsane from inorganic arsenic species with only a minor contribution of dimethylarsane from $DMAs^V$. MAs^V forming methylarsane was a more pronounced interferent in inorganic arsenic species determination but MAs^V is generally absent or only present in trace amounts in rice. However, application of this HG–ICP-MS method on some marine samples was reported to provide overestimated results (compared to HPLC based approaches) due to far more complex arsenic speciation and matrix than rice [7].

The aim of this paper is to present a modified method of HG–ICP-MS with improved selectivity of inorganic arsenic species determination for samples of marine origin (fish and seaweed) whose matrix and arsenic speciation is far more complex than rice matrix.

2. Experimental

2.1 Chemicals and Standards

Deionized water (< 0.2 $\mu S\,cm^{-1}$, Ultrapur, Watrex, USA) 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^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) with Antifoam B (Sigma, USA) was prepared fresh daily. HCl (Merck, Germany) was used for hydride generation. 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 6.0. During optimization of hydride generation, iAs^V water standard was also measured as iAs^{III} after prereduction in 5% KI (Sigma Aldrich, Germany) and 1% ascorbic acid (Riedel-de Haën, China) in 4 M HCl, after 1 h the prereduced solution was 5 times diluted by deionized water. The prereduced iAs^V will be referred to as iAs^{III} hereafter for simplicity.

2.2 Samples

The certified reference materials DORM-3 and DORM-4 (*i.e.,* fish protein), TORT-3 (*i.e.,* lobster hepatopancreas), DOLT-4 and DOLT-5 (*i.e.,* dogfish liver), PRON-1 (*i.e.,* river prawn), and SQID-1 (i.e., cuttlefish) were provided from National Research Council Canada by the courtesy of Dr. Zoltán Mester. Four edible seaweed samples were bought in a local supermarket.

2.3 Sample Preparation

About 200 mg of sample was extracted by 10 cm^3 of 2% HNO_3 and 3% H_2O_2 (10 min ramp 25 to 90 °C and 10 min 90 °C). The microwave-assisted extraction was performed in Milestone Ultrawave system (Italy). After extraction the samples were quantitatively transferred to polypropylene tubes and diluted up to 20 cm^3 with deionized water. All samples were centrifuged at 5000 rpm for 10 min prior to analysis with HG-ICP-MS or HPLC–HG–AFS.

Supernatant was directly analyzed (without any further dilution) by HPLC–HG–AFS. In the case of HG–ICP-MS samples were further two times diluted with 4.5% H_2O_2 (in order to obtain final concentration of H_2O_2 approximately 3%)

2.4 HPLC–HG–AFS

The HPLC–HG–AFS apparatus is described elsewhere [8]. Briefly, a HPLC system Agilent 1200 (USA) was employed, and 50 mm^3 of sample was injected by an autosampler. PRP-X100 (250×4.6 mm, 10 μm particle size, Hamilton, USA) anion exchange column, HG accessories, and the in-house assembled research grade non-dispersive AFS were employed. A miniature diffusion flame under optimum conditions was used as the atomizer [9].

2.5 HG–ICP-MS

The similar HG–ICP-MS setup was used elsewhere [5]. Briefly, the Agilent HG accessory for ICP-MS was used. The sample was injected via an ASX-500 auto-sampler and transported to the hydride generator by a peristaltic pump. The sample was mixed with HCl and $NaBH_4$ in a mixing coil before entering the gas-liquid separator. The reacting mixture was immediately being removed from the gas-liquid separator to the waste by a second peristaltic pump. The gaseous phase was transported to the ICP-MS with an argon gas flow and introduced to a spray chamber of the ICP-MS. A solution of the internal standard (Rh) was nebulized into the spray chamber creating wet plasma conditions. The Agilent ICP-MS 7700x was used for arsenic detection. Measurements were carried in two gas modes (no gas and He) in the collision cell.

3. Results and discussion

3.1 Optimization of conditions for determination

The influence of hydrochloric acid concentration on hydride generation from iAs^{III}, iAs^{V}, MAs^{V}, $DMAs^{V}$, and $TMAs^{V}O$ was investigated in the range from 0.5 to 12 mol dm^{-3}. The main goal was to reach the minimum ratio of sensitivities ($DMAs^{V}/iAs^{V}$) with little emphasis on the ratios of MAs^{V}/iAs^{V} and $TMAs^{V}O/iAs^{V}$

since the MAs^V and $TMAs^VO$ are generally present at trace concentrations. The signals of inorganic arsenic species were constant in whole range of HCl. The gradual decrease of MAs^V signal was observed in the whole concentration range while $DMAs^V$ reached minimum at 8 mol dm^{-3} HCl and then slowly increased. The unusual situation was observed for $TMAs^VO$ because from the concentration of 0.5 to 2 mol dm^{-3} the signal decreased and then started increasing, reaching maximum at 8 mol dm^{-3} HCl (the $TMAs^VO/iAs^V$ ratio was 0.86 ± 0.01). Then the signal decreased to almost zero at 12 mol dm^{-3} HCl.

The concentration range from 0.5 to 3% $NaBH_4$ (in 0.1% KOH) was investigated. The signals of iAs^{III}, iAs^V, MAs^V, $DMAs^V$, and $TMAs^VO$ increased with increasing $NaBH_4$ concentration. The $DMAs^V/iAs^V$ ratio slightly increased at higher $NaBH_4$ concentrations. Therefore, the optimal concentration of $NaBH_4$ (2%) was a compromise between iAs^V sensitivity and $DMAs^V/iAs^V$ ratio.

The addition of hydrogen peroxide increases the extraction efficiency [10]. In this work, it was determined (by potassium permanganate titration) that almost none H_2O_2 is consumed during the microwave-assisted extraction procedure and thus approximately 1.5% of H_2O_2 remains in the final solution (after dilution 1:1 with deionized water). The presence of H_2O_2 may affect the hydride generation from all species. Therefore, the effect of H_2O_2 on hydride generation was tested in the range from 0 to 3%. The signals of iAs^V and MAs^V slightly decreased with increasing H_2O_2 concentration, but the signals of $DMAs^V$ and $TMAs^VO$ were suppressed to a much larger extent.

The contributions of MAs^V, $DMAs^V$, and $TMAs^VO$ to inorganic arsenic species signal were determined from a comparison of sensitivities from the calibrations obtained for each species under optimal conditions of hydride generation (8 mol dm^{-3} HCl, 2% $NaBH_4$, and 3% H_2O_2 were chosen). Numerically, they corresponded to 24.9 ± 0.6%, 0.7 ± 0.1%, and 1.0 ± 0.1% for MAs^V, $DMAs^V$, and $TMAs^VO$, respectively. In order to reduce foaming in the gas-liquid separator which can cause insufficient waste removal and losses of arsanes in the bubbles, 700 mg dm^{-3} of Antifoam B was added to the solution of $NaBH_4$.

3.2 Comparative analyses of seaweed samples and certified reference materials

Inorganic arsenic species contents in DORM-3, DORM-4, TORT-3, DOLT-4, DOLT-5, PRON-1, and SQID-1 certified reference materials and in seaweed samples were determined by HG-ICP-MS method after microwave-assisted extraction. The quantification of inorganic arsenic species in all extracts was performed by external calibration on iAs^V standard in 3% H_2O_2.

The same extracts were also analyzed by an independent method, *i.e.* HPLC–HG–AFS. In this method, mixed calibration standards of $TMAs^VO$ and $DMAs^V$ were used for quantification of iAs^V (due to H_2O_2 used during the extraction all iAs^{III} was oxidized to iAs^V), MAs^V, $DMAs^V$, and $TMAs^VO$. Thanks to 100% hydride

generation efficiency of iAs^V, MAs^V, and $DMAs^V$ all these species could be determined using calibration curve of $DMAs^V$ as we demonstrated previously [8]. The correlation between the concentrations of inorganic arsenic species in certified reference materials and seaweed samples obtained with HG–ICP-MS and HPLC–HG–AFS methods was very good.

4. Conclusions

HG–ICP-MS was optimized to selectively determine inorganic arsenic species, with an insignificant contribution of $DMAs^V$ and $TMAs^VO$. Only MAs^V may contribute significantly, but concentration of MAs^V in real samples was rather low. No significant difference was observed between the results obtained in no gas and helium mode, which means that there was no chloride interference ($^{40}Ar^{35}Cl$) affecting inorganic arsenic species determination. Therefore, there is no need for use of the collision mode which decreases sensitivity and/or an instrument with only a single quadrupole can be practically used as a detector. This method can be used as a fast screening method for large amount of samples, because each sample takes just over 4 minutes for measuring five replicates. This is much shorter compared to speciation analysis based on HPLC which takes 10 minutes or more to obtain only single replicate.

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Miniaturized Boron Doped Diamond Film Electrode for Neuroblastoma Biomarkers Determination

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Abstract
Cathodically pretreated miniaturized boron doped diamond film electrode was successfully tested for the purposes of in-situ determination of neuroblastoma biomarkers after hollow-fibre based microextraction. Electrode was tested using $K_3[Fe(CN)_6]$ and vanillylmandelic acid as model substances. Limits of detection and determination are 2.2 and 6.6 μmol L^{-1}, respectively. Linear range was 2.2–100 μmol L^{-1}, repeatability 1.34% (n = 10).

1. Introduction

Since their introduction in 1990s, boron doped diamond electrodes (BDDE) have become one of the most promising electrode materials, especially for high-sensitivity analytical purposes. BDDEs display many advantageous properties, such as biocompatibility, wide potential window, low background current, excellent mechanical stability, chemical inertness and low susceptibility to fouling/passivation during electrochemical experiments [1, 2]. Moreover, boron doped diamond film can be deposited on a suitable conductive surface to prepare electrodes of various shape and size [3, 4]. Goal of this study is electrochemical characterization of miniaturized boron doped diamond film electrode (BDDFE) deposited on a thin titanium wire. Prospective electroanalytical applications of the BDDFE include sensing in very low volumes of

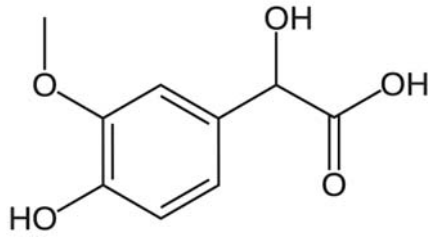

Fig. 1 Chemical structure of vanillylmandelic acid.

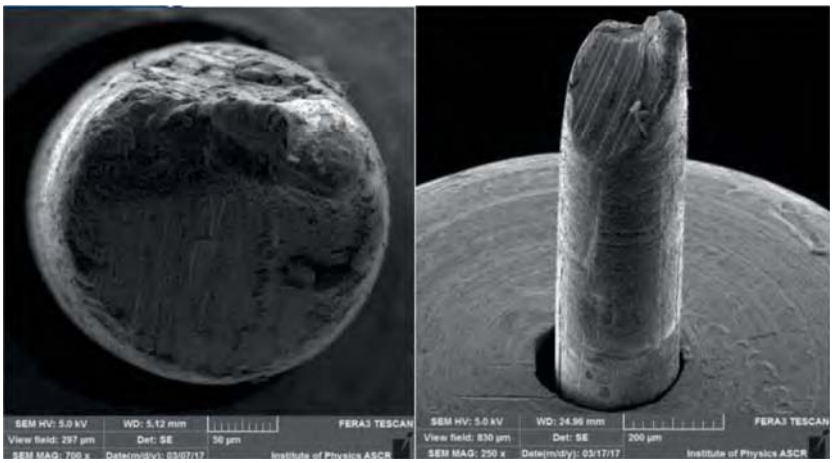

Fig. 2 SEM image of used boron doped diamond film electrode. Boron doped diamond film deposited on titanium wire by micro-wave assisted plasma CVD.

flow-systems or separation fibres for hollow fibre based liquid phase micro-extraction [5, 6].

As a model substance, electrochemically oxidizable vanillylmandelic acid was chosen. Vanillylmandelic acid (Fig. 1) is clinical biomarker of various cancerous, neurological and metabolic diseases, and disorders including neuroblastoma, most common extracranial cancer in infants [7–9].

2. Experimental

2.1 Reagents and chemicals

The stock solution of vanillylmandelic acid (10^{-3} mol L^{-1}) was prepared by dissolving 19.8 mg of solid substance DL-4-hydroxy-3-methoxymandelic acid (≥98%, Sigma Aldrich, Czech Republic) in 100 cm^3 of deionized water. It was stored in dark and cold. $K_3[Fe(CN)_6]$ (≥99%, Sigma Aldrich, Czech Republic). All other chemicals used were at least of analytical grade purity. For all the measurements, deionized water from Milli-Q-Gradient, Millipore, Czech Republic (conductivity < 0.05 µS cm^{-1}) was used.

2.2 Instrumentation

Voltammetric measurements were realized 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). Three electrode system was used with highly doped (equivalent to B/C 2000 ppm) boron doped diamond microelectrode, platinum wire counter electrode and reference Ag/AgCl/3M KCl electrode (both

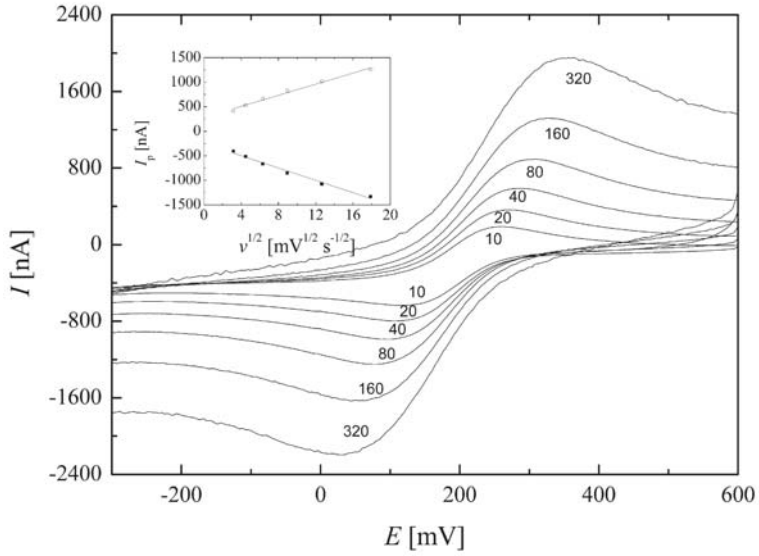

Fig. 3 Cyclic voltammograms of 10^{-3} mol L^{-1} $K_3[Fe(CN)_6]$ in 0.1 M KCl at BDDFE, scan rate 10–320 mV s^{-1}. Corresponding scan rates values are near the curves. Dependencies of I_p / $\upsilon^{1/2}$ in the graph inset.

Monokrystaly Turnov, Czech Republic). Working BDDE was prepared by the microwawe assisted plasma CVD at the Institute of Physics of the AS CR, v.v.i. Boron doped diamond film was deposited on the tip of titanium wire (99.99%, 0.25 mm diameter, Goodfellow Cambridge, United Kingdom; Fig. 2) seeded with standard Osawa dispersion, time of deposition 5 h.

2.3 Procedures

BDDFE was cathodically pretreated before each measurement by applying potential of −1000 mV for 15 s. Differential pulse voltammetry (DPV) was carried out applying scan rate of 20 mV s^{-1}, the pulse amplitude 50 mV, pulse duration 100 ms, sampling time 20 ms beginning 80 ms after the onset of the pulse and interval between pulses of 100 ms. The limit of quantification (*LOQ*) was calculated as $LOQ = 10\ s/a$, where s is the standard deviation of 10 repetitive measurements of the lowest measurable concentration and a is the slope of the calibration curve. The limit of detection (*LOD*) was calculated as $LOD = 3.3\ s/a$.

3. Results and discussion

The BDDFE was characterized by cyclic voltammetry, using 10^{-3} mol L^{-1} $K_3[Fe(CN)_6]$ in 0.1M KCl as a redox probe (Fig. 3). Well-developed peak and counter-peak couple was observed at 150 mV and 240 mV. The difference between peak and counter-peak potentials was higher than theoretically

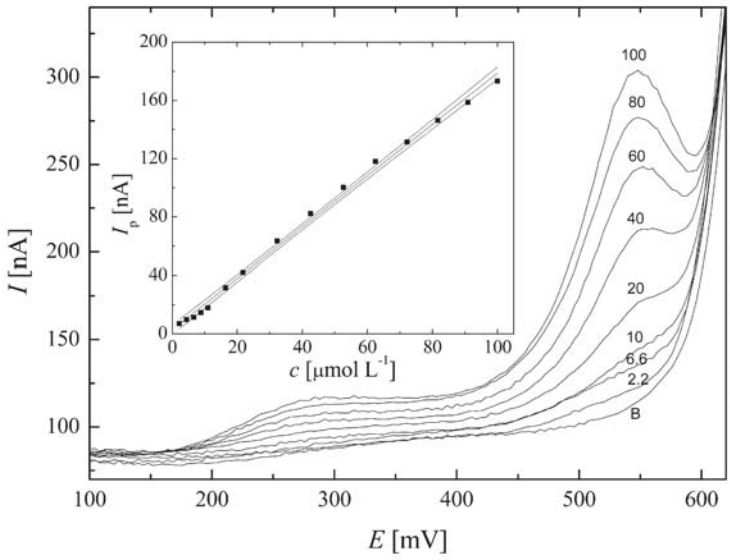

Fig. 4 DPV curves of 0–100 μmol L^{-1} vanillylmandelic acid at BDDFE in 0.1 M NaOH. BDDE cathodically pretreated at –1000 mV for 15 s. Corresponding vanillylmandelic acid concentrations are displayed near curves, 0.1 M NaOH blank sample (B). Inset shows linear dependency of the peak current of vanillylmandelic acid on the concentration.

predicted 59 mV, indicating quasi-reversible process. Shift of the peak potential towards more negative (in reverse scan to more positive) values was also observed as scan rate increased, confirming quasi-reversible or irreversible nature of process. As can be seen in inset of Fig. 3, dependence of peak potentials on the square root of the scan rate is linear, indicating possible diffusion controlled process

$$I_{p,\mathrm{Red}}\,[\mathrm{nA}] = (-246.4 \pm 34.51)\,[\mathrm{nA}] + (-62.78 \pm 3.37)\,\upsilon^{1/2}\,[\mathrm{mV\,s^{-1}}] \quad (1)$$
$$R = -0.992$$

$$I_{p,\mathrm{Ox}}\,[\mathrm{nA}] = +(281.5 \pm 32.89)\,[\mathrm{nA}] + (56.89 \pm 3.21)\,\upsilon^{1/2}\,[\mathrm{mV\,s^{-1}}] \quad (2)$$
$$\mathrm{R} = +0.992$$

However, the criterion is inconclusive, due to non-planar shape of the used electrode and large observed intercepts of the $I_p / \upsilon^{1/2}$ dependencies.

Due to intended future use of this method for vanillylmandelic acid determination inside a separation fibre after liquid/liquid/liquid hollow-fibre microextraction, 0.1M NaOH (pH = 13) was chosen as a solvent to ensure the highest possible enrichment factors of the extraction [7].

For determination of vanillylmandelic acid, which is negatively charged under basic conditions of 0.1M NaOH, mild cathodical pretreatment (−1000 mV for 15 s) was applied before each measurement to avoid repulsion of the O-terminated

Table 1
Parameters of concentration dependency of vanillylmandelic acid at BDDE.

Parameter	Tested BDDFE	Commercially available BDDE [7]
Linear range / μmol L^{-1}	2.2–100	1.2–100
LOD / μmol L^{-1}	2.2	1.2
LOQ / μmol L^{-1}	6.6	3.6
RSD (n = 10) / %	1.34	1.8

BDDFE surface and the analyte. Such repulsion leads to substantial and completely undesirable shift of the vanillylmandelic acid peak potential towards more positive values. Effects of BDDE pretreatment are discussed in article by Schwarzova at al. [10].

The relative standard deviation (*RSD*) of peak currents (I_p) after 10 consecutive DPV determinations of 100 μmol L^{-1} vanillylmandelic acid was found to be 1.34%, suggesting very good repeatability.

DPV concentration dependencies of vanillylmandelic acid were measured in the range of 1–100 μmol L^{-1}, the dependency was linear in the range of 2.2–100 μmol L^{-1} (Fig. 4) and is desribed by the following equation

$$I_p\,[\text{nA}] = (2.92 \pm 1.195)\,[\text{nA L mol L}^{-1}] + (1.75 \pm 0.03)\,c\,[\mu\text{mol L}^{-1}] \quad (3)$$
$$R = 0.999$$

Limit of detection (*LOD*) and quantification (*LOQ*) were found to be 2.2 μmol L^{-1} and 6.6 μmol L^{-1}. Due to higher background noise, LOD and LOQ of vanillylmandelic acid at the BDDFE are slightly higher compared to commercially available bulk BDDE with 3 mm diameter (Table 1).

4. Conclusions

Miniaturized BDDFE was successfully employed for determination of vanillylmandelic acid under basic conditions suitable for three phase hollow-fibre based microextraction of vanillylmandelic acid. Due to its small size, BDDFE can be inserted inside the separation fibres for in-situ detection. Calculated repeatability, LOD and LOQ are comparable to those obtained using a substantially larger commercially available boron doped diamond electrode and sufficient for determination of vanillylmandelic acid in human urine samples.

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