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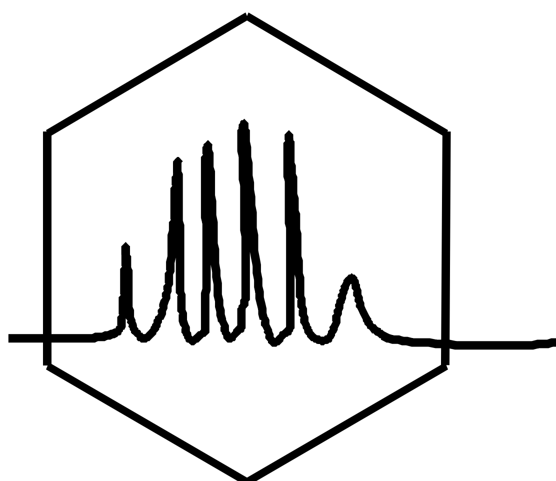

THE JUBILEE XXXth SYMPOSIUM

'CHROMATOGRAPHIC METHODS OF INVESTIGATING THE ORGANIC COMPOUNDS'

JUNE 12th – 14th, 2006
KATOWICE – SZCZYRK

BOOK OF ABSTRACTS

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PLENARY SESSION I

HPLC WITH GRADIENT OF pH OF THE MOBILE PHASE AS A METHOD OF EFFICIENT DETERMINATION OF ACIDITY AND LIPOPHILICITY OF XENOBIOTICS

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Our recent year research (1-5) resulted in an original method of reversed-phase high-performance liquid chromatography (RP HPLC) employing programmed gradient of pH of the mobile phase. The gradient is developed to efficiently separate acidic or basic analytes. Unlike the standard gradient RP HPLC mode, where the increase of elution strength of the eluent is due to an increase of the content of organic modifier, in our method the increase of elution strength of the mobile phase is due to programmed increase (in the case of acidic analytes) or decrease (in the case of basic analytes) of the pH of aqueous eluent.

The pH gradient mode of RP HPLC appeared technically feasible using the modern stationary phases. In a series of reports we have presented a comprehensive mathematical model of RP HPLC retention using gradient pH of the eluent. The model allows prediction of changes in retention of a given analyte resulting from the changes of chromatographic conditions and, eventually, optimization of separations. It also allows to determine pK_a value of the analyte.

A combination of two gradients: pH and concentration of organic modifier, provides a unique chromatographic procedure of simultaneous determination of acidity and lipophilicity of analytes. The method allows a high-throughput screening (HTS) and rational preselection of "drug candidates", which are now often prepared in small amounts and as multicomponent mixtures, without a need to separate individual compounds.

The approach proposed by us, which is based on a strict theoretical model, consists in retention measurements in a series of RP HPLC runs at a wide range of methanol concentration gradient differing in the range of the simultaneously developed gradients of pH and the time of gradient. The method has been verified on a representative group of 93 acidic and basic drugs. It was demonstrated that reliable pK_a data are obtained within the range of 3-10 units and lipophilicity parameter (log P) within 0-7 units.

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PLENARY SESSION I

CHARACTERIZATION OF COLUMN SELECTIVITY FOR USE IN TWO-DIMENSIONAL LC SEPARATIONS

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The main objective of two-dimensional LC separations is to increase the peak capacity, i.e., the number of sample compounds that can be separated in a single run. For this purpose, efficient columns and orthogonal systems should be used with large differences in selectivity in the two dimensions for various polar and non-polar structural elements in a sample.. A model was derived for the 2D system enabling to predict the degree of orthogonality from the correlation between the polar and lipophilic selectivities in the 1st D and 2nd D systems and the range of polar or lipophilic structural units that can be resolved in a given time range in a two-dimensional sample with different structural unit distribution. Gradient elution or programmed temperature can significantly improve the overall peak capacity in a 2D system.

In comprehensive two-dimensional LC, every part of sample is subject to separation on the columns both in the first and in the second dimension. For this purpose, short or monolithic columns enabling fast separation within one minute or less are very advantageous. A crucial point for successful sample transfer between the first and in the second dimension is the selection of compatible stationary phase chemistry and mobile phase in the two dimensions. Further, various sample transfer modulation techniques can be used to maintain the resolution accomplished in the first dimension by utilizing auxiliary sample enrichment columns instead of sampling loops or on-column sample focusing effect.

A hydrophilic interaction LC system in the second dimension for RP-NP LCxLC systems is more resistant against aqueous solvents transferred in the fractions from the first, RP dimension, than an NP system with a non-aqueous mobile phase. Combinations of bonded C18, polyethyleneglycol and zirconia/carbon columns offer almost orthogonal systems for separations of various polar samples. Applications for comprehensive 2D separation of natural phenolic antioxidants and of EO-PO (co)oligomers are shown.

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PLENARY SESSION I

ψ-SPECTRA – A NEW TOOL FOR SUBSTANCE IDENTIFICATION IN CHROMATOGRAPHY

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During the analysis of residues or contaminants a positive signal in terms of retention characteristics has to be checked also in terms of compound identity. In the field of liquid chromatography (HPLC, TLC), substance identification is usually performed by electron spectroscopy, besides mass spectrometry actually an up-coming technique for TLC, too [1, 2].

However, the information to be obtained from absorption spectra is quite low with respect to fine structure and slope. This information can be drawn out from the raw data by derivatization, but the signal heights of derivative spectra are still concentration dependent. To overcome this problem we introduced so-called ψ-spectra calculated by deviding the derivative spectrum by the original absorption spectrum ($\psi^n = D^n/D^0$) and, therefore, eliminating the dependence upon concentration. One can say, the information of ψ^n is 'only' the characteristic slope at each point of the spectrum.The method was originally developed for spectrum purity checks during photometrical analyses [3], but was recently adopted to HPLC/DAD analyses.

In contrast to transmission measurement used in HPLC, recording of a spectrum on a HPTLC plate is performed by measurement of the diffuse reflection. The absorption spectrum of a substance is obtained after correction of the initially recorded spectrum by both the background spectrum and the lamp spectrum. Although proper background correction is a crucial factor of influence for spectrum comparison, spectra are almost comparable from plate to plate allowing the use of spectra libraries.

However, the spectrum quality/identity of TLC spots is generally known to be dependent on concentration (absolute amount on a spot), while in HPLC normalized spectra from different standard runs are completely identical. To what extent derivative and ψ-spectra improves spectral differentiation on a planar chromatogram is the aim of present studies.

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HPTLC-ESI/MS, HPTLC-ESI/MS/MS AND HPTLC-DART/TOF-SUITABLE FOR MASS CONFIRMATION OF POSITIVE FINDINGS IN TRACE ANALYSIS?

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Coupling of column chromatography with mass spectrometry is a current hyphenated technique in trace analysis and contributes to the success of these methods to a large extent. Regarding planar chromatographic methods a major disadvantage was the lack of coupling possibilities with mass spectrometry. Recent approaches for extraction or desorption of substances directly from a HPTLC plate enable sensitive mass spectrometric signals within one minute or even within seconds.

HPTLC-ESI-MS and HPTLC-ESI-MS/MS by a plunger-based extraction device [1, 2] is shown to be an appropriate coupling technique for quantitative planar chromatography. Extraction from silica gel phases, the most important separation material in planar chromatography, and sensitivity in the lower pg-range were important features which distinguishes this technique from other approaches. Its possibility for employment in trace analysis is demonstrated [3, 4].

Recently DART (Direct Analysis in Real Time) was introduced [5]. This kind of versatile new ion source is working in open air under ambient conditions. The employment of DART in the field of planar chromatography was successfully demonstrated in initial studies [6]. Within seconds the mass spectrum of a substance can be obtained provided that the substance of interest is positioned on the plate edge. Nevertheless plate positioning seems to be a crucial factor regarding reproducibility and analytical response.

In our opinion a unique advantage of coupling HPTLC with MS is the minimal employment of the MS equipment due to the local fixation of separated substance zones on the planar chromatogram: after quantification, only positive findings can be confirmed via MS and not every chromatographic run is recorded a priori.

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PLENARY SESSION II

EFFECT OF EXTRACTION METHOD ON THE YIELD OF SECONDARY METABOLITES FROM PLANT MATERIAL

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Sample pre-treatment is typically one of the most time-consuming steps of the analytical process, particulary when solid samples are invloved, e.g. solvesnt extraction of solid samples. Among the techniques used for this step, exhaustive extraction in Soxhlet apparatus is one that has frequently employed for more than a century. Nowadays it is also main reference by which the performance of other leachnig methods may be compared. There are, however, significant drawbacks associated with Soxhlet extraction such as the long time required for extraction and the large amount of solvent required and other. In terms of extraction efficiency, recent leching methods are better owing to the use of solvents at high temperature an pressure – ultrasonification (USAE), microwave assisted solvent extraction (MASE), accelerated solvent extraction – pressurised solvent extraction (ASE or PLE). These techniques require shorter extraction time, and less solvent can be used.

In our investigations we have examined the following substances and plant materials: furanocoumarins in fruits of Archangelica officinalis and Pastinaca sativa, taxoids in twigs of Taxus species, flavonoids and phenolic acids in flowers of Sambuccus nigra, flavonoids in herb of Polygonum avicculare, tropane alkaloids in herb of Datura innoxia.

We have found that in case of furanocoumarins the highest yield of extraction was obtained by PLE method with the simultaneous low time- and solvent consmption. Also USAE and MASE in open system give satisfactory results. The use of MASE in pressurised system can not be applied for the isolation of furanocoumarins because of decomposition of some substances.

Similar conclusions can be drawn from the experiments of extraction of taxoids from twigs of Taxus sp. The highest yield was obtained by PLE with methanol in time 10-15 min at 130^0C. Simultaneously the results were repeatable with low RSD values.

In case of flavonoids and phenolic acids from Sambuccus nigra flos the highest yield was obtained by Soxhlet extraction. However, when the same substances were extracted from the herb of Polygonum aviculare exhaustive extraction in Soxhlet apparatus had low efficiency. Highest yield was obtained by PLE and other microwave- or ultrasound assisted techniques. It can be caused by the differences in anatomic structure of plant tissues.

Also modern extraction techniques were effective for the extraction of alkaloids from the herb of Datura innoxia in comparison to percolation.

INVERSE GAS CHROMATOGRAPHY AND PRINCIPAL COMPONENT ANALYSIS IN CHARACTERIZATION OF POLYMERIC COMPOSITIONS

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Compositions of polymers (Polyethylene, Polyuretane) and fillers (initial silica and silicas modified with: N-2-aminoethyl-3-aminopropyltrimethoxysilane, 3-aminopropyltriethoxy-silane, 3-merkaptopropyltrimethoxysilane, n-octyltriethoxysilane) were examined by inverse gas chromatography at 383K. Small amounts of the following test solutes were injected to achieve the infinite dilution conditions: pentane, hexane, heptane, octane, nonane, dichloromethane, chloroform, carbon tetrachloride, and 1,2-dichloroethane.

The retention times for these test solutes were determined and Flory-Huggins parameters were calculated. Flory-Huggins parameters characterizing interactions between components: χ_{12}^{∞} - between composition and the test solute and $\chi_{23}^{'}$ - between polymer and filler in the composition.

Values of both physicochemical parameters characterizing the examined materials were arranged in a matrix form: in the rows the compositions were numerated at different temperatures whereas the columns contained the test solutes. After standardization the input matrix was subjected to principal component analysis. Three principal components explain more than 93% of the total variance in the data. Almost all test solutes carry very similar information. Therefore, it is justified to eliminate most of them from the series of test solutes.

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PLENARY SESSION III

DETERMINATION OF POTENTIALLY TOXIC ORGANIC COMPOUNDS EMITTED FROM RESIN-BASED DENTAL MATERIALS BY MEANS OF HS-SPME-GC-MS AND HS-SPME-GC PROCEDURES

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Leaching of volatile substances from resin-based dental materials may have a potential impact on the biocompatibility as well as safety of these materials. Information from manufacturers on ingredients in the materials is very often incomplete. Patients and dentists may be in contact with components emitted from cured dental fillings or from substrates applied in their preparation. Therefore, determination of the components of these materials is necessary for better prevention from possible harmful effects caused by dental fillings. The aim of this work was the isolation and identification of organic compounds evolved from four commercial resin-modified glass-ionomer cements (resin-based dental materials applied in dentistry) by using an alternative method of volatile compounds analysis - HS-SPME (headspace-solid phase microextraction). Dental materials were heated in closed vial at various temperatures and volatile substances evolved into the headspace phase above sample were isolated into thin polymeric fibre placed in SPME syringe. Identification was performed by using of GC-MS (gas chromatography-mass spectrometry) technique. Almost 50 RMGICs (resin-modified glass-ionomer cements) components (monomers and additives) were identified. The main identified leachables were: iodobenzene (DPICls - diphenyliodonium chloride degradation product), camphorquinone (photo-initiator), tert-butyl-p-hydroxyanisole (inhibitor), 4-(dimethylamino) ethyl benzoate (co-initiator), ethylene glycol dimethacrylate (monomer).

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PLENARY SESSION III

VALIDATION OF ANALYTICAL METHOD IN THE LIGHT OF VARIOUS REGULATIONS AND A PRACTICAL APPROACH

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Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Method validation has received considerable attention in literature and from industrial committees and regulatory agencies. The Guidance on the Interpretation of the EN 45000 Series of Standards and ISO/IEC Guide 25 includes a chapter on the validation of methods [1] with a list of nine validation parameters. The International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use [2] has developed a consensus text on the validation of analytical procedures.

Validation of chromatographic methods of well-characterised systems, such as are found in the pharmaceutical industry, is based on a series of experimental procedures to establish: selectivity/specificity, accuracy, precision (repeatability, reproducibility), linearity, detection limit, quantitation limit, range, stability, and robustness.

A rapid, accurate and sensitive reversed-phase high-performance liquid chromatographic (RP-HPLC) method has been developed for the determination of different drugs in biological samples by our colleague and validation parameters has been experimentally received.

The terms selectivity and specificity are often used interchangeably. The term specific by ICH generally refers to a method that produces a response for a single analyte only while the term selective refers to a method which provides responses for a number of chemical entities that may or may not be distinguished from each other. Selectivity in liquid chromatography is obtained by choosing optimal columns and setting chromatographic conditions, such as mobile phase composition, column temperature and detector wavelength. Precision was determined by using different levels of drug concentrations studying repeatability and inter-mediate precision. Repeatability (%RSD) was below 10% at all three levels of concentrations. Repeatability results indicate the precision under the same operating conditions over a short interval of time and inter-assay precision. Intermediate precision expresses within-laboratory variations in different days, what corresponds with quality control at three concentrations. In intermediate precision study, RSD values were not more than 15 % in all cases. RSD values were within the acceptable range indicating that these methods have excellent repeatability and intermediate precision. Accuracy was assessed as the percentage relative error and mean percentage recovery. To give additional support to accuracy of the developed assay method, standard addition method was done. In this study, different concentrations of pure drug were added to a known preanalysed biological sample and the total concentration was determined using the proposed method (n=3). The percent recovery of the added pure drug was calculated. The linearity of an analytical method is its ability to elicit test results that are proportional to the concentration of analytes in samples within a given range. Linearity is determined by a series of six or more injections of standards. A linear regression equation applied to the results should have an intercept not

significantly different from zero. The range of an analytical method is the interval between the upper and lower levels that have been demonstrated to be determined with precision, accuracy and linearity using the method as written. The limit of detection is the point at which a measured value is larger than the uncertainty associated with it. It is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified. In chromatography the detection limit is the injected amount that results in a peak with a height at least twice or three times as high as the baseline noise level. The limit of quantitation is the minimum injected amount that gives precise measurements, in chromatography typically requiring peak heights 10 to 20 times higher than baseline noise. If the required precision of the method at the limit of quantitation has been specified, the EURACHEM [1] approach can be used. A number of samples with decreasing amounts of the analyte are injected six times. The calculated RSD% of the precision is plotted against the analyte amount. The amount that corresponds to the previously defined required precision is equal to the limit of quantitation. Stability was confirmed by freezing and thawing test which did not reveal any detrimental effect on the absolute concentrations of analyte spiked to human plasma/serum and investigated at three different concentrations. After completion of three freezing and thawing cycles, the measured concentrations of drug still ranged between 85 and 105%. Robustness tests examine the effect operational parameters have on the analysis results. For the determination of a method's robustness a number of chromatographic parameters, for example, flow rate, column temperature, injection volume, detection wavelength or mobile phase composition are varied within a realistic range and the quantitative influence of the variables is determined.

In summary, the our proposed methods were simple, rapid, accurate, precise with satisfactory reproducibility, stability, specific, selective and can be used for routine analysis of different drugs as well as for bioavailability studies.

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PLENARY SESSION III

TEMPERATURE EFFECTS IN LIQUID CHROMATOGRAPHY

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The main objective of this research communication is influence of temperature on chromatographic separation under TLC and HPLC conditions, focusing mainly on the author and co-workers approach. Application of elevated and sub-ambient temperatures for separation of compounds like calixarenes, cyclodextrins, macrocyclic antibiotics, flavones, fullerenes, prostaglandins, polycyclic aromatic hydrocarbons as well as wide range of steroids including cholesterol, bile acids, estrogens, progestogens, ginsenosides and stress hormones will be reported [1-7]. Moreover number of problems important from practical point of view will be discussed particularly:

a) Application of vertical and horizontal TLC chambers for non-forced planar chromatography in different temperatures.
b) Solubility of β-cyclodextrin and hydroxypropyl β-cyclodextrin in selected binary water-organic liquids at elevated and sub-zero temperature
c) Non-linear behaviour of the freezing point profile of the mobile phases based on aceto-nitrile/water mixtures.
d) Separation capability of mobile phases unmodified and modified with macrocyclic modi-fiers that are non-retarded by stationary phase ligands including α, β, γ-cyclodextrins and their hydroxypropyl derivatives.
e) Thermodynamic studies of the retention behaviour of steroids, polycyclic aromatic hydrocarbons and macrocyclic compounds based on TLC and HPLC retention data.

Furthermore a simple optimization strategy for the separation of multicomponent mixtures from biological samples especially for metabolomic investigations using temperature as the critical parameter for selectivity in the liquid chromatography will be demonstrated.

Acknowledgements

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THERMODYNAMIC DESCRIPTION OF RETENTION IN REVERSED-PHASE LIQUID CHROMATOGRAPHY

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Two different molecular processes, i.e. displacement (adsorption) and partition, are usually used in reversed-phase chromatography to represent the solute's distribution between the mobile and stationary phase. Displacement is a surface process which occurs at the solid-liquid interface whereas partition involves solutes transfer from the bulk eluent into stationary phase.

The partition model is especially useful to description of RPC retention of small nonpolar solutes in the organic modifier range from 0-70%. Molecular mechanism of partition process involves: (1) the creation of solute-sized cavity in the stationary phase, (2) the transfer of a solute molecule from the mobile phase to the formed cavity in the stationary phase and (3) the closing of a solute sized cavity in the mobile phase. As should be evident, the solute's partitioning is controlled by the differences in the molecular interactions of solutes in the stationary and mobile phases. So, it is possible to calculate free enthalpy of retention (ΔG_R^o) as a sum of the change in free enthalpy due to solvent effects and stationary phase effects.

Based on liquid-crystalline hydrocarbon model of stationary phase free of solvent molecules we obtained expression for the retention factor:

$$\log k = x_w \log k_w + x_o \log k_o + \frac{-\Delta G^o_{cav}}{2.303RT} + \frac{-\Delta G^o_{\mathrm{int}}}{2.303RT} + \log V - \log \Phi$$

where k, k_w and k_o are the retention factors in mixed eluent, water and organic modifier, x_w and x_o are molar fraction of water and organic modifier, ΔG^o_{cav} is the free enthalpy of creation cavities in pure solvents and closing cavity in mixed eluent and ΔG^o_{int} is the free enthalpy of solute-solvents interactions.

All chromatographic data were obtained using the Shimadzu Vp liquid chromatographic system equipped with LC 10AT pump, SPD 10A UV-VIS detector, SCL 10A system controller, CTO-10 AS chromatographic oven and Rheodyne injector valve with a 20 µL loop. This system utilizes class-Vp computer program to control hardware, acquire and store data and determine retention time.

The stainless-steel column (12.5 cm × 4 mm, I.D.) packed with LiChrospher RP-18e, a particle diameter 5 µm (Merck) was used in each experiment. Mixtures of methanol-water were used as effluents. The methanol molar fraction ranged from 0.1 to 0.9 (in steps of 0.1) for most solutes. Nonpolar and week polar benzene derivatives were used as tested substances.

PLENARY SESSION IV

CARDIOLIPIN, ITS DEACYLATION IN MAMMALIAN MYOCARDIA. A MINI REVIEW. A CHROMATOGRAPHIC-COMPUTATIONAL ANALYSIS

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We have developed a key protocol that utilizes multiple thin layer chromatographic (TLC) techniques and different mobile phases coupled with wavelength-specific densitometry, and computational analysis. This combination permits the quantitative recognition of diverse phospholipids and their respective metabolic products resulting from the in vitro activation of their endogenous phospholipases.

Cardiolipin (CL) occupies a unique position in the metabolism of phospholipids that is not yet well defined.

In an attempt to learn more about the actual role of CL and its in vitro deacylation phenomenon in mammalian myocardia, we conducted an in vitro incubation of whole tissue homogenate as source of phospholipases and phospholipids of heart from diverse mammals.

Resulting TLC-densitometric analysis of the in vitro response of these tissues will be reported, as well as their correlation with the in vivo metabolic situation.

CHEMICALLY MODIFIED VOLCANIC TUFF FOR TLC APPLICATIONS

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The volcanic tuff, a rock formed by deposition of material expelled during volcanic eruptions, is a natural adsorbent with chromatographic applications. Its composition depends on the material origin and any transformation occurring at one time. The percentage oxide composition of the volcanic tuff from Mârşid, Romania, is: 67.2 SiO_2, 11.4 Al_2O_3, 0.8 Fe_2O_3, 3.3 CaO, 1.1 MgO, 0.3 Na_2O, 3.3 K_2O and 0.2 TiO_2. Due to its high silanol content, this material is proper to obtain chemically modified stationary phases.

In this paper, the unmodified volcanic tuff and chemically modified by organosilylation reaction with n-alkyl (C8, C10, C12, C18), 3-aminopropyl, 3-(2-aminoethyl-amino)propyl and 3-mercaptopropyl trifunctional silanes are studied.

By FTIR spectroscopy, the presence of modifier on volcanic tuff surface was evidenced. New weak bands were observed in the 2800–3000 cm^{-1} range due to the ν(CH) vibrations of CH_2 and CH_3 groups. To improve the sensibility of infrared method, the difference and the second derivative spectra were also analyzed.

The conformational properties of immobilized ligands were studied by ^{13}C-CP/MAS NMR spectroscopy. Silane functionality and bonding chemistry were determined by ^{29}Si-CP/MAS NMR spectroscopy. These spectra indicated a high cross linking for all samples and a different surface coverage of them.

The thermoanalytical (TG, DTG, DTA) method gives quantitative information about the temperature ranges where thermal effects take place due to some processes like the removal of water by desorption (endothermal effect), the dehydration and the elimination of organic part from modifier (exothermal effects).

The TLC behavior of the chemically modified volcanic tuff was tested by the separation of different compounds such as: dyes, organophosphorous pesticides, benzopyrene derivatives, biologically active compounds.

Our studies show that as a result of the chemical modification of the volcanic tuff surface with different modifiers, the hydrophilic properties of its surface decreased, the volcanic tuff being converted into new stationary phases, having a different degree of hydrophobicity, according to the nature of the modifier.

PLENARY SESSION IV

APPLICATIONS OF HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY IN FOOD AND PHARMACEUTICAL ANALYSIS

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High performance thin layer chromatography (HPTLC) is a widely used separation technique for rapid screening of several kinds of compounds. HPTLC has relevant capabilities that make it appropriated for routine work. It is an off-line separation system that enables simultaneous evaluation of standards and samples, thus matching the working and environmental conditions for both, reducing the systematic errors, also allows a fast quantification up to 36 - 72 samples simultaneously with the possibility of multiple detection by multi-wavelength scanning. The clean-up process is less exhaustive, than in the other types of chromatography, because the plate is discarded after analysis, which allows a rapid work, especially in complex matrixes such as plants and food, with an enormous saving of time.

In this presentation, will be showed the applications of HPTLC in food and pharmaceutical analysis, especially focused in analysis required by the Chilean industry. Among these, the lecture will include: antibiotic and antibacterial detection in salmon feed, astaxanthin detection in salmon feed, mycotoxins analysis in cereals, starch in flour and premixes, sugars in wines and plants, available lysine in foods, biogenic amines in fishmeal, lipids fraction in egg's yolk, cholesterol in cheese, complex B-vitamins, caffeine and taurine in energy drinks and lecithin and ascorbic acid in powdered milk. With regards to pharmaceutical compounds, the detection of drugs with single component, as carbamazepine, benzodiazepine and metamizol and drug with multiple-components, as headache medicines will be showed. Also some analysis using an online HPTLC-ESI/MS system will be included. The objective of this lecture is to show, through the analyses normally asked by the industry, the versatility and reliability of the HPTLC system for routine analysis.

CHARACTERISATION AND SELECTION OF REVERSED-PHASE LIQUID CHROMATOGRAPHIC COLUMNS

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Official compendia such as the European Pharmacopoeia (Ph. Eur.) and the United States Pharmacopeia (USP) often prescribe liquid chromatographic reversed-phase analyses. Since they are not allowed to mention brand names of columns, they only give some general information about the stationary phase to be used. This information is usually insufficient to select a suitable column from a market offering several hundreds of types.

In this study, a test procedure to determine 36 chromatographic parameters (selected from the literature) was evaluated on 69 different columns. 24 out of the 36 parameters measured were found to be repeatable and reproducible. Column classification was performed using principal component analysis (PCA). A similar classification could be obtained by reducing the number of chromatographic test parameters to 4 [1-4]. Based on these 4 parame-ters, a column ranking system versus a (freely to choose) reference column has been elaborated [5].

In a next step, the correlation between the test parameters and the separation characteristics was investigated in practice for separations of different drug substances and their impurities [6,7]. Most of the separations were performed according to the prescriptions of the respective Ph. Eur. monograph. The chromatographic response function was used to evaluate the separations. Nice results were obtained. Besides, the predicting value of the system suitability test prescribed in the monographs was also evaluated.

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PLENARY SESSION V

NEW APPLICATIONS OF RECURRENT RELATIONSHIPS IN ORGANIC CHEMISTRY AND CHROMATOGRAPHY

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Recently it was shown that variations the most physicochemical constants of organic compounds (A) within homologous series can be described by linear (first order) recurrent relationships [1, 2]:

$$A(n+1) = a\,A(n) + b \qquad (1)$$

The existence of this regularity provides the simplest way to precalculate the values of any properties (including chromatographic constants) of higher homologues using data for previous members of series. The following law has been postulated: most of known constants of organic compounds depend linearly upon their values for previous homologues.

Only one exception from regularity (1) has been found. Strong alteration effects (differences in properties for compounds with even and odd number of carbon atoms in the molecules) are usually displayed in melting points (T_m). The approximation of these data by first order recurrent equation (1) gives two unequal linear sub-dependencies for every subset of compounds. However, the application of second order recurrent equation (2) provides the single relationship, as it can be seen on the example of alkanecarboxylic acids (Fig. 1):

$$T_m(n+2) = a\,T_m(n) + b \qquad (2)$$

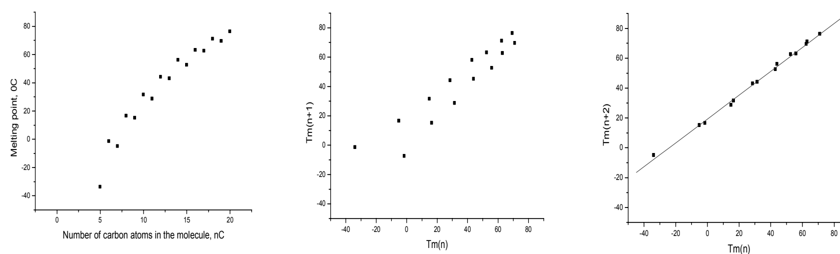

Fig. 1. Dependencies of melting points (T_m) of alkanecarboxylic acids ($C_nH_{2n+1}CO_2H$) upon number of carbon atoms in the molecule (non-linear, left); first order recurrent dependence $T_m(n+1) = a\,T_m(n) + b$ (two sub-lines for homologues with even and odd number of carbon atoms, central), and single second order linear recurrent dependence $T_m(n+2) = a\,T_m(n) + b$ (right); $a = 0.799 \pm 0.014$, $b = 19.3 \pm 0.6$, $\rho = 0.998$, $S_0 = 1.5$.

Linear recurrent dependence (2) is observed for transition temperatures of liquid crystals (Prof. E. Sokolova, St. Petersburg State University, private communication), as well.

Recurrent regularity (1) permits us to propose absolutely new approaches for solution of typical chromatographic problems (i.e., estimation of retention parameters of higher homologues using the data for previous members of series) instead of well known linear dependence

$\log (t_R – t_0) = a\, n_C + b$, where $t_0 \approx (t_{R2}^2 - t_{R1} \times t_{R3}) / (t_{R1} + t_{R3} – 2t_{R2})$ and t_{R1}, t_{R2}, t_{R3} – retention times of three consecutive homologues.

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PLENARY SESSION V

CLASSIFICATION OF SAMPLES USING CHROMATOGRAPHIC DATA

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Chromatographic techniques offer a broad scope of possible applications and allow acquiring interesting information for studies samples. Later, this information is used in order to extract chemically relevant knowledge about the samples by means of various chemometrical techniques. The data analysis is performed either in supervised or unsupervised manner. In supervised data modelling, except a set of measurements requires, additional information about the data samples is available and thus the goal of the analysis is to construct classification or calibration models [1]. Such external information about the samples could be, for instance, geographical origin of samples, a certain physicochemical property. Using a set of measured parameters, the classification models give a possibility to predict to which class of samples a new sample belong, whereas the calibration models predict a certain property of interest. The unsupervised data analysis is entirely based on the set of measured parameters and there is no additional information about the samples. For unsupervised data analysis usually different projection [2] and clustering techniques [3] are used to visualize and explore the data structure.

Here, we would like to present a relatively new and simple chemometrical approach, called CAIMAN (Classification And Influence Matrix ANalysis) [4], very well suited for classification problems. A main advantage of CAIMAN is that it is conceptually simple, very fast and capable to derive classification rules for linear and non-linear classification problems. Moreover, different variants of CAIMAN are available allowing discrimination of samples, modelling of the classes and modelling a selected class being very far from all other ones.

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PLENARY SESSION V

NOVEL SAMPLE HANDLING TECHNIQUES IN GAS CHROMATOGRAPHY TO ENHANCE DETECTION LIMITS AND IMPROVE PERFORMANCE AND RELIABILITY

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The monitoring of many analytical samples requires sensitive and selective methods as many legislations are becoming more stringent every year.

To meet these sensitivity demands the use of automated sampling techniques is of help to concentrate the samples and analyze them in unattended operations.

This talk presents several new applications developed with the use of an enhanced headspace autosampler that uses an on-board packed trap. This headspace trap is a new technology that is capable of sampling up to 100% of the headspace by a pulsed-pressure headspace extraction process with analytes refocused on an adsorbent trap. The trap is then rapidly heated to desorb the trapped analytes and carrier gas sweeps them via an optional splitter as a narrow band onto the GC column. There are substantial gains in sensitivity as a result of the increased sample volume injected (up to 100x in some instances).

Other applications will demonstrate the use of a new line of thermal desorption systems that have been significantly enhanced with over 30 new features including redesigned programmable pneumatics, sample recollection technology, system and sample integrity tests.

These features allow the user to repeat the analysis from a single tube and we will demonstrate their application in the analysis of ambient air and in some food sample.

PLENARY SESSION VI

USE OF HPLC IN NONDENATURING CONDITIONS FOR THE STUDIES OF DNA QUADRUPLEX FORMATION AND DNA-LIGAND INTERACTIONS

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Guanine- rich oligonucleotides can form a variety of multistranded structures known as G-quadruplexes, guanine-tetraplexes or G4 DNA [1]. The main interest in G-quadruplexes is due to their potential links to mechanisms that relate to cancer therapy involving telomeric DNA. Inhibition of telomerase activity in cancer cells is believed to be possible through the formation of guanine quadruplex structures on the telomeric DNA strand [1]. The quadruplex is stabilized by metal cations coordinated in a central channel of G-quartet (Fig. 1A). For example, the sodium quadruplex formed by the human telomeric DNA sequence, d[AGGG(TTAGGG)$_3$], possesses antiparallel strand orientation with one diagonal and two lateral TTA loops at the G-quartet ends (the basket-type structure – Fig. 1B).

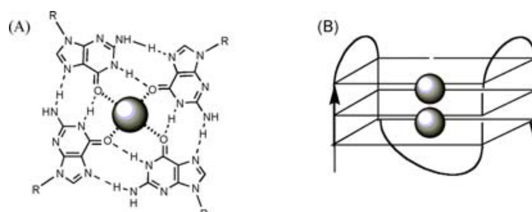

Figure 1. (A) Structure of G-quartet showing hydrogen bonds between four guanines and the interaction with a cation (circle). (B) Schematic representation of the antiparallel "basket-type" G-quadruplex with one diagonal and two lateral loops

Interestingly, the intramolecular potassium complex with human telomeric sequence exhibits a variety of G-quadruplex structures ranging from antiparallel basket- or chair-type structures to a controversial parallel conformation called also a propeller-type structure. The overall topologies available to G4 structures and their polymorphism depend on many factors including strand stoichiometry and polarity, glycosidic torsion angle, connecting loops, and coordination of cations [1].

Several techniques are employed to explore formation and properties of G-quadruplexes including gel electrophoresis, X-ray diffraction, mass spectrometry, NMR and other molecular spectroscopy techniques.

Here, we try to evaluate HPLC separations in nondenaturing conditions for detection of different structures of G4 DNA in the presence of metal cations. An approach to use HPLC for monitoring DNA-ligand interactions is also reported.

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LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY FOR THE CHARACTERIZATION OF IMPURITIES IN VANCOMYCIN AND DIRITHROMYCIN

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Antibiotics are essential and life-saving drugs. There is for the moment an interest and uprise in the search for new antibiotics, able to conquer the problem of increasing resistance to existing antibiotics. The quality of all pharmaceuticals, including antibiotics, needs to be monitored continuously, to safeguard therapeutic success and ascertain the absence of toxic components. In this study, emphasis was put on the characterization of unknown substances present in the antibiotics vancomycin and dirithromycin. Hyphenated techniques such as liquid chromatography/mass spectrometry (LC/MS) are ideally suited for the purpose of impurity characterization. As a general strategy, a library was built of fragmentation patterns of the main compound and available reference substances. Indeed, knowledge of these fragmentation patterns allows to understand the structures of unknown related substances, established by comparison with these data.

Vancomycin is a glycopeptide antibiotic, with strong bactericidal activity against many Gram-positive bacteria. It can be used in penicillin-allergic patients. It is a natural product of Amycolatopsis orientalis, and several structurally related compounds are formed during the fermentation process. In this study, the structures of six unknown substances occurring in commercial samples were elucidated. These substances were identified as demethylleucinevancomycin B, 26-acetyldecarboxyvancomycin B, desamidovancomycin B, N-demethylvancomycin B, N-methylvancomycin B and 20-chlorovancomycin B.

Dirithromycin is a semi-synthetic oxazine derivative of erythromycin, that exhibits antibacterial activity against a variety of Gram-positive and Gram-negative bacteria. Recently, LC showed the presence of several impurities of unknown identity in a dirithromycin sample. In total nine impurities were elucidated by LC/MS.

EVALUATION OF CAPILLARY ELECTROCHROMATOGRAPHY AS CHIRAL SEPARATION TECHNIQUE – DEFINITION OF A GENERIC SEPARATION STRATEGY

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The separation of chiral molecules is an extensively studied field in pharmaceutical analysis because enantiomers can exhibit different properties in human systems. Regulatory instances therefore demand the development of separation methods, in order to determine the enantiomeric purity and stability of chiral drugs. Because the separation of these drug molecules often requires extensive method development, the definition of generic separation strategies, i.e. applicable on large sets of structurally diverse compounds and giving separation for most of them, can be very useful.

Capillary electrochromatography (CEC) was evaluated for its potential as analytical separation technique to define such separation strategy. CEC combines the properties of both high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE), and is characterized by its miniaturization, high efficiencies, fast separations, and a high sensitivity when a mass spectrometer is used as detector.

Chiral separations in CEC were investigated using both direct approaches, i.e. adding a chiral selector to the mobile phase or using chiral stationary phases (CSP). Numerous experiments allowed to conclude that the use of the latter was suitable for our aims. Because of their broad enantioselectivity, four polysaccharide CSP were finally selected to define the strategy: Chiralcel OD-RH and OJ-RH, Chiralpak AD-RH and AS-RH. Separate strategies were defined for acidic compounds on the one hand, and basics, bifunctionals and neutrals on the other. The strategies were constructed as decision trees: first a general screening experiment is executed, and depending on the obtained separation, occasional optimization is performed.

The proposed strategies were tested on 15 acidic and 48 basic, bifunctional and neutral compounds. In total, enantioselectivity was observed for 82.5 % of the test set after application of the strategies, indicating their potential in chiral method development.

ANTIOXIDANT CAPACITY OF GREEN TEA EXTRACTS ESTIMATED FROM CHROMATOGRAMS USING PARTIAL LEAST SQUARES REGRESSION AND A COLORIMETRIC REFERENCE METHOD

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[2]The University of Silesia, Institute of Chemistry, Katowice, Poland

In this study, a fast strategy for determining the total antioxidant capacity of Chinese green tea is developed. This strategy includes the use of experimental techniques, such as fast High-Performance Liquid Chromatography on monolithic columns and a spectrophotometric approach to determine the total antioxidant capacity of green tea. To extract the chemically relevant information from the obtained data, chemometrical approaches are used. Among them there are Correlation Optimized Warping to align the chromatograms, Robust Principal Com-ponent Analysis to detect outliers and Partial Least Squares and Uninformative Variable Eli-mination Partial Least Squares for modeling. These approaches allow to process the data and to construct a reliable multivariate regression model between the chromatographic and spectro-photometric data for the prediction of the total antioxidant capacity of green teas from fast chromatograms.

Reference:

A.M. van Nederkassel, M. Daszykowski, D.L. Massart, Y. Vander Heyden, "Prediction of total green tea antioxidant capacity from chromatograms by multivariate modeling", Journal of Chromatography A, 1096 (2005) 177-186.

PLENARY SESSION VI

LIQUID CHROMATOGRAPHIC DETERMINATION OF GLYOXAL AND METHYLGLYOXAL FROM SERUM OF DIABETIC PATIENTS USING MESO- STILBENEDIAMINE AS DERIVATIZING REAGENT

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Meso-stilbenediamine has been used as derivatizing reagent for liquid chromatographic (LC) determination of glyoxal (Go), methylglyoxal (MGo) and dimethylglyoxal (DMGo) at pH 3. Liquid chromatographic elution and separation was carried out from the column Kromasil 100 C-18, 5µm (15 x 0.46mm id) with methanol : water : acetonitrile (59 : 40 : 1 v/v/v) with a flow rate of 1ml/min. and UV detection at 254 nm. The linear calibrations curves were obtained for Go, MGo and DMGo within 0.97-4.86µg/ml, 1.52-7.6µg/ml and 1.41-7.08µg/ml with detection limits of 48ng/ml, 76ng/ml and 70.8ng/ml respectively. The method was applied for the determination of Go and MGo from serum of patients suffering from diabetics and ketosis. The amount of Go and MGo found were 0.150-0.260µg/ml and 0.160-0.270µg/ml with coefficient of variation 2.6-4.7 %and 2.5-4.6 % respectively. The results obtained were compared with normal subjects with Go and MGo contents of 0.025-0.065µg/ml and 0.030-0.070µg/ml with C.V 1.5-4.9 % and 1.6-4.8 % in the serum.

INVERSE GAS CHROMATOGRAPHY AND PRINCIPAL COMPONENT ANALYSIS IN CHARACTERISATION OF FILLERS USED IN ABRASIVE ARTICLES

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Abrasive articles consists of cutting particle – very often from electrocorundum, filler – inorganic compound, e.g. potassium fluoroborate or cryolite, binder – novolak resin and wetting agent - resol. The most important stages during manufacturing of grinding tools are: the coverage of the abrasive by wetting agent and proper hardening. The fillers play a very important role during the work of the grinding tools – they collect the heat and prevent the melting of resin. The surface properties of the components of grinding tools influence the interactions between them and the properties of the final product.

Surface properties of different kinds of the fillers were examined by means of Inverse Gas Chromatography (IGC). Dispersive component of the surface free energy γ_S^D at relative humidities: 30, 60 and 90% and at various temperatures: 278, 283, 288 and 293K was determined. It provided the data on stability of the fillers during their storage and/or weathering. The influence of the type of the filler on the hardening process by differential scanning calo-rimetry (DSC) was also studied.

Principal Component Analysis (PCA) was applied to search the influence of the temperature and humidity onto the γ_S^D values for various fillers. This analysis showed that changes of the surface properties of fillers are significant at higher humidity close to 90%. Furthermore, PCA demonstrated that the type of filler does not influence meaningfully the hardening process.

This approach has shown usefulness of IGC and calorimetric methods in studying the properties of abrasive articles as well as PCA in the interpretation of physicochemical charac-teristics of abrasive materials.

This work was supported by Ministry of Education and Science grant Project No 3 T09B 051 29 what is gratefully acknowledged.

THE PRINCIPLE OF MOLECULAR RECOGNITION FOR THE DETERMINATION OF RESIDUAL DRUGS FROM SERUM USING MACROCYCLIC AGENTS

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The application of macrocyclic agents (β-cyclodextrin, β-cyclodextrin sulfated salt (S-β-CD), 4,13-diaza-18-croun-6) – synthetic analogues of recognition systems as components in chromatographic or electrophoretic conditions allows to realize the selective and effective separation of different organic compounds from biological objects (serum, plasma, urea, структура мозга). It caused by macrocycle unique ability for complex formation «host – guest» type. Stabilization is carried out by different non-valency interactions (hydrophobic, ion-dipole and other).

The strategy of macrocycle choice considerable determined by the nature of defined components, composition of buffer system or mobile phase.

The methods of capillary zone electrophoresis, micellar electrokinetic chromatography, reversed phased high performance liquid chromatography and high performance thin-layer chromatography with UV-detection were used in this investigation for the selective separation and quantitative determination of steroidal and non-steroidal drugs.

Fig. 1 presents the inclusion complex of β-cyclodextrin with on of the drug – trimipramine.

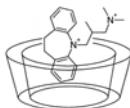

Fig. 1. The interaction of drug trimipramine with β-cyclodextrin

The addition of S-β-CD into the buffer solution allows the concurrent separation of nature steroids (cortisol, corticosterone) and steroid drug – prednisolone using the mode of capillaryzoneelectrophoresis (Fig. 2).

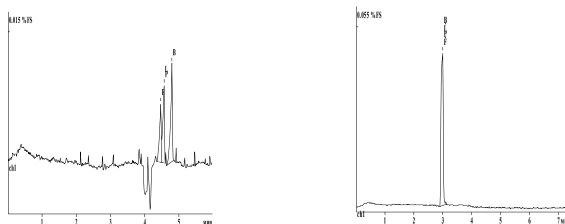

Fig. 2. Electropherogram of steroids model sample (0,3 µg/ml): F – cortisol, PL – prednisolone, B – corticosterone.
<u>Equipment:</u> Capel 103 (Lumex Ltd. Russia), L_{tot} =60 см, L_{eff} =50 см, $d_{I.D.}$=75 µm. <u>BGE</u>: 6,25 mM borate buffer, pH 9,3 1) without addition S-β-CD, 2) with 5 mM S-β-CD

CHEMICAL VARIANCE: ON THE INTERPRETATION OF TWO-DIMENSIONAL CHROMATOGRAPHIC DATA

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A new method is presented for the analysis of comprehensive two-dimensional data. The approach complements existing chemometric methods for analysing these kinds of data (e.g. multivariate-curve-resolution methods, MCR), and is especially suited for the analysis of families of compounds. The method is based on finding a new pair of axes (replacing the retention time axes) to represent the data. A drop in the matrix rank is observed when one of the axes collects all the variance for a certain family of compounds. This is achieved by introducing retention models (in both chromatographic dimensions) that describe the behaviour of a family of compounds as a function of the property that varies between the members. After this transformation, a single family of compounds gives rise to a single source of variance. This suggests the application of MCR techniques in a second step to separate the contributions of different (overlapped) families of compounds. For the latter application, more research has to be performed.

The approach is illustrated using the separation of families of functional poly(methyl-methacrylate) polymers with different numbers of hydroxyl end groups and the degree of polymerization or, equivalently, the molecular weight as the key descriptive property within one family.

CHIRAL SEPARATIONS USING KNAUER EUROCEL 01 FROM ANALYTICAL UP TO PROCESS SCALE

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Eurocel 01 (cellulose-tri(3,5-dimethylphenylcarbamate)) is a versatile, new and attractively priced alternative to current chiral stationary phases (CSPs), available from KNAUER for analytical, preparative, SMB (simulated moving bed), and even process scale applications.

As a polysaccharide-based CSP, Eurocel 01 can be used to separate approximately 80% of all chiral compounds and can be applied in normal phase (NP), reversed phase (RP) and polar organic (PO) modes. Eurocel 01 offers one of the highest loadabilities of all CSPs along with high separation power, high enantioselectivity and a pressure resistance comparable to that of regular silica.

Current studies of the chiral separation of racemic phenyl alcohols and pyrrole-imidazole alkaloids have demonstrated the high eluent versatility of Eurocel 01. Without the need for high buffer concentrations, Eurocel 01 CSP can be used in reversed phase mode to achieve reproducible results over a long period.

For preparative chromatography Eurocel 01 is available in large packed columns or as bulk material. Preparative and process scale HPLC is required for the production of chiral compounds if stereoselective synthesis is either not possible, insufficient or simply too expensive. SMB systems are particularly suited to the separation of binary mixtures, as is typical in most chiral applications. Additional advantages of SMB systems include high productivity due to continuous (as opposed to sequential) operation, very efficient use of the stationary phase and low consumption of mobile phase (about 90 % less). Very small and flexible SMB systems based on the patented KNAUER multiport valve can produce up to 100 or 1000 kg/year. Upscaling from analytical conditions is made simple with KNAUER's IsothermFit® and SMB_Guide® software.

THE CHROMATOGRAPHIC METHOD OF HYDROGEN CONCENTRATION MEASUREMENT IN THE AIR

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In response to continuous population growth and the industrialization of developing countries, the world's demand for energy is continuosly increasing, but the resources of fossil fuels are limited. The scientist are searching the alternative sources of energy.

One of the solution is hydrogen economy. The using of hydrogen as a source of energy in fuel cells will take place all around where the electricity is necessary (for consumer electronics like laptop computers, cell phones, audio players etc).

Unfortunatelly, application hydrogen in fuel cells can have some consequences for the atmosphere. The higher concentration of hydrogen may indirectly lead to higher concentration of methane, which is one of the major greenhouse effect gasses.

In the Laboratory of Environmental Physics Institute of Nuclear Physics Polish Academy of Science in Kraków the chromatographic method of hydrogen measurement in the air (with the use of a Fisons gas chromatograph) was designed. The air sample is analysed in two chromatographic columns filled with a molecular sieve of type 5A working in the "back-flush" mode. To the hydrogen detection was used Pulsed Discharge Helium Ionisation Detector.

The presented method will be used for hydrogen monitoring in air.

This work is partly supported by grand no. 4T12B 047 28, 4T12B 004 28 and 3T09D 038 293 from the Ministry of Education and Science.

METHOD OF CHROMATOGRAPHIC MEASUREMENT OF HELIUM CONCENTRATION IN GROUNDWATER FOR HYDROLOGICAL PURPOSES

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Key words: gas chromatography, hydrology, noble gasses, helium, groundwater dating, head-space method

Helium concentration in groundwater can be an indicator of groundwater age. If the local helium accumulation rate can be determined, it may be possible to estimate the ages of waters that exceed 100 years, up to tens of thousands of years. Helium concentration in the atmosphere is about 5.24 ppm. Knowing helium solubility in water, one can calculate the concentration of helium in surface water using the Henry's law. Surface water in equilibrium with the atmosphere and at the temperature of 20^oC contains about $8.6 \cdot 10^{-12}$ gHe/cm^3 of water. Helium concentration can be increased in groundwater as a result of excessive air getting into the water or as a diffusion and accumulation in water radiogenic helium.

A chromatographic system of measurement of helium concentration in groundwater using the head-space method is presented. The chromatograph used in this method is of Shimadzu make, equipped with the TCD detector. Helium extracted from the water sample is enriched in a trap immersed in liquid nitrogen. The trap is made of stainless steel and filled with active charcoal. Helium accumulated at the top of the trap is injected into three chromatographic columns working in the "back flush" mode and detected by the TCD detector (with detection limit of about $1.6 \cdot 10^{-12}$ g He/cm^3 of air). Water samples are taken from groundwater with a precise procedure without contamination with air. These samples are collected in a special glass vessels of volume equal to 1180 cm^3.

This work was accomplished as part of grants No. 4T12B 047 28, 4T12B 004 28 and No. 3T09D 038 29 from the Ministry of Education and Science.

DETERMINATION OF POLYCHLORINATED BIPHENYLS IN GASES FROM THE PYROLYSIS OF PVC

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Resource recycling of the plastic material wastes by thermal methods consists in destructive conversion of the polymers contained in these materials to the low molecular compounds. The pyrolysis plants for the disposal of wastes are generally preferred to the combustion plants as safer to the environment. Nature, composition, and quantity of the resulting degradation products depend on many factors, the most important among them being the kind and chemical composition of wastes, the maximum range of the temperatures applied, and the duration time of the pyrolysis. [1,2]. In pyrolytical gases, there can appear – depending on composition of the processed wastes – along with CO, H_2, CH_4, and HCl [3], polychlorinated biphenyls (PCBs) as well.

In this study, a newly elaborated method was presented of determination of polychlorinated biphenyls in gases from the pyrolysis of PVC. This method was based on the GC/ECD technique, and the linear ranges of detection and the lowest levels of determination of the nine PCBs were established. The way of sample preparation for the GC analysis was elaborated, including extraction from the sorbent material and the cleaning of the extract.

In order to estimate the content of PCBs in the gases originating from the pyrolysis of the PVC wastes, the pyrolysis tests were performed. In these tests, the quartz retort was applied, in which the ground PVC wastes (the average pellet size 3 mm) were placed. The pyrolysis of the PVC wastes was carried out in an inert atmosphere of argon, flowing through the reaction vessel with the speed of 0.39 L min^{-1}. Samples of pyrolytical gases were collected during the process running at the temperature of 500^0C by adsorption on the XAD-2 resin. Further analysis of the gas for the content of PCBs was carried out by means of the GC/ECD method, using the capillary column DB-5MS (30 m × 0.25 mm × 0.25 µm).

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LONGITUDINAL TRANSECTS OF METHANE CONCENTRATION IN ATMOSPHERE OF SOUTHERN POLAND

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Southern territory of Poland includes exceptionally large number of methane sources both anthropogenic and natural origin. Silesia region, which is known as the biggest mining district of Poland, extends on several hundreds of kilometers in latitudinal direction. Ventilation systems of mines deliver into the atmosphere constant flux of diluted methane. Methane ema-nates also through the cracks of bedrock causing increase of atmospheric concentration in temporary local scale up to several hundreds of ppm. City gas network supplying few millions of people still has lot of leakages, what can affect methane concentration on urban area. Additionally waste landfills produce meaningful methane amount at the most urbanized region of Poland. Typical methane production rate in Silesia mining area was parallel to the coal excavation and was reported in year 1985 to about 1Mt C per year and remains at the level of 0.5 Mt C per year nowadays.

Methane analyzer "Gascorder" produced by Baseline Inc. corporation was installed on board of car driven through the investigated area with average speed 75km/h. Inlet of air was in the front of the car avoiding self contamination of measurements by car exhausts or air condition. Container with 25L of dry carbon dioxide was used as cryogenic trap what allowed 300km of transect to be done without maintenance of equipment. During all transects position of car was recorded with GPS in same intervals as concentration readouts (usually 10 sec). Parallel to the transects methane concentration was monitored in Kraków and at Kasprowy Wierch. However principle of Gascorder work bases on FID detector without separation of sample it requires frequent calibration with the standard mixtures and comparison with results of spot samples taken simultaneously and measured by GC.

Average variation of methane concentration during transects is usually not larger than 400 ppb in region not industrialized and exceeds 2500ppb in mining and industry districts. As transects were performed during relatively short time (4h) in midday, when atmosphere was unstable and well mixed in summer, average methane concentration in months V – IX was increased by 150ppb in compare to reference free troposphere Kasprowy Wierch. Also analyses in Kraków revealed slightly higher values (approx 50 – 100ppb). During the summer months methane concentration in lower troposphere of great part of Southern Poland is comparable to free troposphere. In so called Upper Silesia region methane concentration is increased by 400ppb even when meteorological condition implies intensive mixing of atmosphere. Transect performed in winter months (X – IV) revealed significant difference between lower tropospheric CH_4 mixing ratio and Kasprowy Wierch record. As the temperature inversion lasts for the few days methane concentration increases in this time to values 2400ppb even in places where there is no sources of this gas.

Night time transects conducted during the stable condition of atmosphere even for summer months discovered areas where methane concentration raised over 10000ppb particu-larly close to mine ventilation exhausts (in radius of 1km).

Both spatial and temporal distribution of methane may be used by carbon budget models to verify source pattern and model kinetics.

CHROMATOGRAPHIC FINGERPRINT DEVELOPMENT FOR HERBS: A SCREENING AND OPTIMIZATION HPLC/UV METHODOLOGY ON MONOLITHIC COLUMNS

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All over the world, herbs are used for preventive and therapeutical goals. Therefore, identification and quality control of products of natural origin is required. Determination of a number of active compounds does not allow assessing the total intrinsic quality of herbs. Since 1991 the World Health Organization accepts fingerprint chromatography as identification and quality evaluation technique for medicinal herbs. In fingerprint development, the first step in the process is to create general conditions to enlarge the peak capacity with an acceptable resolution.

To propose a methodology to optimize fingerprint chromatograms in HPLC/UV, four herbs, Liquorice, Artichoke, Cascara and Curcuma, are screened with several organic modifiers in order to define a gradient elution with an optimal selectivity. Monolithic C18 columns were used as stationary phases. Further, a sequential approach was applied to select the most optimal conditions for wavelength, column length, flow rate and slope of the gradient for developing the best fingerprint.

To evaluate the global quality of a fingerprint chromatogram, different parameters such as the sum of Resolutions (Rs), the Hierarchical Chromatographic Response Function (HCRF), the Chromatographic Response Function (CRF) and the Chromatographic Optimization Function (COF) were compared and the applicability of those parameters as decision criteria was examined.

MODEL OF STYRENE DISTRIBUTION IN POLYSTYRENE FOAM

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Styrene is known as a cancer precursor specious and therefore its emission from building insulation materials is restricted and should be quantified according to standardized methods. Thus polystyrene foam is usually aged after production for certain time. However styrene is endlessly permeating from interior to surrounding environment. There are only few work describing styrene diffusion (permeation) through polymer material and almost none in low temperatures under glass transition temperature. In this temperature polystyrene is not thermally destructed however styrene preserved in the cells can be efficiently released. Laboratory test were prepared carefully with polystyrene blocks sized 2cm x 5cm x10cm and with helium as flushing headspace gas. The experiments were conducted in different temperatures between 25 and 90 degrees of Celsius.

To interpret the data obtained from the headspace measurement (GC-MS) computational model was created. It resolves numerical diffusion equations and mass balance in 3D space. Calculation procedures base on inverse modeling protocol and use experimental data to compare with model result, fit the parameter of model and recalculate the equation to obtain the lowest cost function value.

Agreement of model results with experimental data achieved in higher temperatures is surprisingly good (average relative deviation remains under 0.05). Time constant of emission process as well as spatial and temporal distribution of styrene in polystyrene block may be observed in real time simulation. Spatial gradients of styrene concentration depends strongly on temperature. Several assumption simplifying the process of permeation was applied to the model equations resulting discrepancy of data and model in lower temperatures as well as decreasing of sensitivity to the diffusion coefficient. Model has high sensitivity to adsorption and desorption coefficients which are represented with satisfied accuracy. This constants computed by the model are in good agreement with literature data.

Model was prepared in Matlab computation environment.

ALIPHATIC ALCOHOLS – IS THEIR CONSIDERABLE POLARITY AN OBSTACLE IN MEASURING MOLAR VAPORIZATION ENTHALPIES BY MEANS OF CAPILLARY GAS CHROMATOGRAPHY OR NOT?

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This paper is a consecutive one in the series devoted to the non-classical approaches to determination of thermodynamic data. In our earlier studies, we have tested alkylbenzenes [1], aldehydes [2], and ketones [3] in an analogous way. Presently, we focus our attention on aliphatic alcohols, which are the analyte group even more polar than the preceding ones.

In our current work – following an earlier idea [4-7] – we made use of the capillary gas chromatography (CGC) as a measuring tool, combined with the selected mathematical and statistical procedures. As a result, gas chromatography once again proved applicable to physicochemical investigations, and more specifically – it demonstrated good performance as a handy alternative for microcalorimetry.

With aid of gas chromatography the simple retention data (i.e. the retention times) can be collected, as an empirical basis for the specially devised mathematical equations, derived from the physicochemical theory and from the semi-empirical chromatographic models. These equations appear as the one- or two-parameter linear or exponential relationships. The respective fitting parameters have a physicochemical meaning and from the numerical values thereof (derived, owing to the selected statistical procedures) certain thermodynamic data can be estimated.

Ten equations that describe the relationships between the retention parameters (i.e., relative retention, retention coefficient, and the Kováts retention index) on the one hand and the selected physical magnitudes of the test alcohols (i.e., boiling points, molar volumes, and molar refraction) on the other were examined to show their ability to predict the analytes' retention and their selected thermodynamic characteristics. The thermodynamic magnitudes derived from our computations were the molar enthalpies of vaporization (ΔH_{vap}) of the test alcohols and the standard chemical potentials of partitioning of one methylene group between the stationary and the mobile phase in a given chromatographic system ($\Delta\mu_{p(\text{-CH2-})}$). Performance of our thermodynamic estimations was assessed by a comparison of the respective numerical values derived from our models with those originating from the literature.

The chromatographic measurements were carried out isothermally on the stationary phases of different polarity, at five different working temperatures.

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CGC AS AN ACCURATE TOOL FOR DERIVATION OF THE HEAT CAPACITY VALUES WITH ALIPHATIC ALCOHOLS. DOES THE EXPERIMENT CONFIRM THIS STATEMENT OR OTHERWISE?

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In paper [1] we introduced a non-classical approach to determination of the heat capacities and tested it with use of the three groups of organic compounds (i.e. of alkylbenzenes, aldehydes, and ketones). Instead of a traditional, tedious, and rather complicated calorimetric determination of this magnitude, we proposed a novel approach, based on certain semi-empirical equations. These mathematical equations couple retention parameters of an analyte with simple physical magnitudes, in that way being a compilation of physicochemical theories and certain chromatographic models. Our approach enables estimation of the numerical values of heat capacity in an indirect and computationally rather entangled manner, although the experiment itself is incomparably simpler than in the case of calorimetry. In fact, the only experimental data necessary for the further processing are the retention times of analytes, measured under the well defined working conditions.

The chromatographic retention data used in this study originate from isothermal measurements performed for aliphatic alcohols (as a novel group of the test analytes) with a selection of stationary phases of different polarity at five different working temperatures. Then – following the computational procedure – the numerical heat capacity values were calculated and finally, these results were compared with the two data sets. One data set was taken from literature and originated from the direct (i.e. calorimetric) measurements. The other one was derived computationally, using the Kopp and Neumann Rule.

In this study we scrutinized usefulness of the mathematical equations and at the same time applicability of the examined procedure to derivation of the heat capacities with the group of aliphatic alcohols. Once again it was clearly shown that the proposed approach results in a satisfactory enough outcome of the reliable heat capacity values.

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ORTHOGONAL PROJECTION APPROACH TO SELECT DISSIMILAR CHROMATOGRAPHIC SYSTEMS

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Developing an analytical separation procedure for an unknown mixture is a difficult issue. An important example is the quantification of a new drug and its impurities, which is required by the International Conference on Harmonization [1]. One approach helping to solve this problem is the use of dissimilar chromatographic systems showing large selectivity differences to screen the substances. After the screening the most suited system for further method development can be chosen. The first step of this strategy consists of defining a dissimilar set of chromatographic systems. In order to create a fast selection procedure, which allows an easy evaluation of new commercially available stationary phases, Orthogonal Projection Approach (OPA) was tested [2]. Initially "retention spectra" were constructed plotting the retention times as a function of the corresponding compounds, which were then compared by the dissimilarity criterion of OPA. The selected spectra, i.e. the most dissimilar, show the largest differences in retention times for a set of compounds. This means that the corresponding chromatographic systems show selectivity differences and can be considered dissimilar. From this study, it could be concluded that OPA is able to select a set of orthogonal chromatographic systems. The selection differs slightly from those obtained by other evaluated techniques [3-5], but that can be explained by differences in the selection criteria. OPA is a fast, easy and objective method. The largest drawback is that no groups of similar systems are determined. However several other techniques applied for this purpose [3-5] suffer from the same drawback.

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STUDY OF RETENTION PARAMETERS OBTAINED IN RP-TLC SYSTEM AND THEIR APPLICATION ON QSAR/QSPR OF SOME ALPHA ADRENERGIC AND IMIDAZOLINE RECEPTOR LIGANDS

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The role of lipophilicity in drug-biomacromolecule interactions has been extensively discussed in terms of quantitative structure-property, and structure-activity relationships (QSPR/QSAR). The objective of this work is to investigate the hydrophobicity parameters obtained in RP-TLC system, and a possible application in QSAR studies of imidazolines, ami-dines and related drugs.

The retention constant, Rm^0, is determined for eleven selected adrenergic and imida-zoline-based receptor ligands by RP-TLC. The correlation among the following parameters: hydrophobic parameters Rm^0, log P, and electronic, geometric, topological and molecular size-related descriptors obtained with multi linear regression (MLR) in two- and three-parameter analysis is investigated.

Satisfactory correlations are found between the following: (1) Rm^0 and apparent partition coefficient octanol/buffer pH 7.4 (log P'), and (2) apparent partition coefficient in four liposome systems (log K'M) and hypotensive activity (pC_{25}) for five imidazolines.

The results validate that the proposed approach is suitable for QSAR/QSPR studies of these drugs.

CHROMATOGRAPHIC ANALYSIS OF THE SELECTED AMINOACIDS ON THE CHEMICALLY BONDED STATIONARY PHASES IMPREGNATED WITH THE TRANSITION METALS CATIONS. PART II

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This study is a continuation of our previous efforts aimed at establishing of the most convenient working conditions for separation of amino acids. The ready made TLC glassplates covered with the chemically bonded stationary phases of the RP-8 and RP-18 type were impregnated with the salts of metals such as Cu(II), Cd(II), Co(II), Ni(II), Mn(II) and Al(III), Cr(III), Sr(III). Six aminoacids were analysed on the above mentioned nonimpregnated and impregnated stationary phases by use of a variety of experimentally selected mobile phases. The values of the retardation parameter R_F and of the geometrical index Ig were calculated.

The results obtained in this study allow to us notice that impregnation has a strong effect on the values of the retardation parameter R_F of the six analysed amino acids. For most of them the range of the R_F values obtained on the impregnated layers was wider than on the non-impregnated ones. Furthermore, in many cases impregnation enables separation of the analysed amino acids and even allows to obtain more compact spots of analytes.

THE INVESTIGATION OF THE INFLUENCE OF THE IMPREGNATION OF THE CHEMICALLY BONDED STATIONARY PHASES ON THE RETENTION MECHANISM OF THE SELECTED AMINO ACIDS

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In this paper we present the results from our recent studies focusing on the influence of the impregnating agents on the retention mechanism of the selected amino acids. Six of them were chromatographed on the non-polar RP-2 and RP-8 chemically bonded stationary phases, non-impregnated and impregnated with chiral compounds (such as L-ascorbic acid and L-tartaric acid). The values of the retardation parameter R_F and of the geometrical index Ig were calculated.

The results of our studies indicate that in most cases the value of the retardation parameter R_F is generally higher on the impregnated stationary phases than on the non-impregnated ones. In addition, it can be observed that impregnation affects solute retention giving the possibility to get the well-shaped and compact spots and what is even more important, to chromatographically analyse selected amino acids which on the same, but non-impregnated phases do not even start to develop.

APPLICATION OF ARTIFICIAL NEURAL NETWORKS TO CHROMATOGRAPHIC DATA ANALYSIS

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Application of chromatographic techniques in atmospheric greenhouse gas measurements requires high accuracy and reproducibility. One of the most important steps in data analysis is integration of peak area. In this study, the artificial neural network (ANN) technique was applied to this step. The ANN was trained using artificial data and then was used to calculate peak area in real chromatograms. Obtained data are compared to calculations done by standard integration procedure available in Agilent ChemStation software. The comparison shows that reproducibility of both methods is similar (Fig 1), but ANN technique is more stable in case of variation of analysis conditions. Additionally the detection limit can be improved by this approach significantly. A series of tests with artificial data containing chromatographic peaks with different signal to noise ratio shows that calculation of peak area gives satisfactory results (correlation between expected and calculated value grater than 0.8) down to signal to noise ratio value of 0.3.

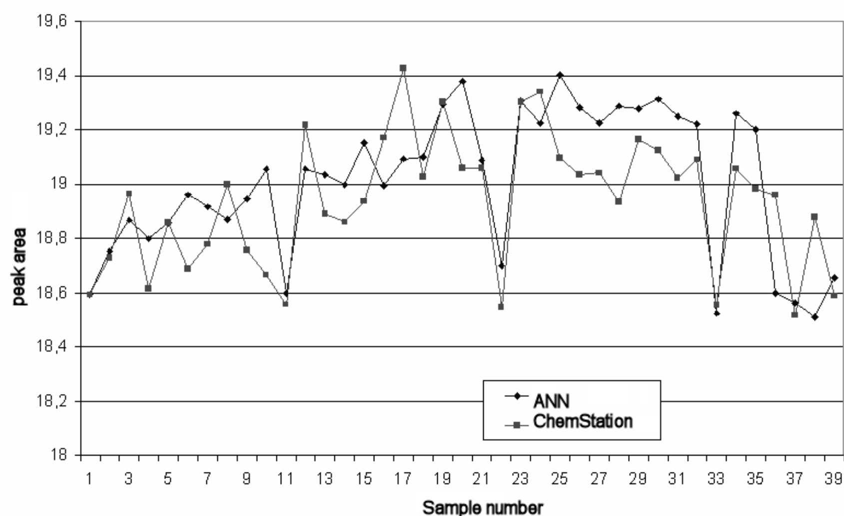

Fig. 1 Comparison of the peak area obtained from ChemStation procedure and ANN calculation

log *P*, log k_w AND ϕ_o AS HYDROPHOBICITY INDEX OF SOME FUNGICIDES

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Hydrophobicity or lipophilicity is one of the most important properties determining biological activity of compounds like drugs, herbicides or fungicides. The logarithm of partition coefficient in n-octanol/water system, log *P*, is generally used as a hydrophobicity index, but different chromatographic parameters, e.g. log k_w – the logarithm of solute retention parameter in water or ϕ_o – the concentration of the mobile phase corresponding to log $k = 0$, are proposed as alternative descriptors of hydrophobic properties of organic substances as well. Because these parameters can be measured experimentally very rarely in practice log *P* values can be evaluated using theoretical procedures while log k_w parameters are calculated by extrapolation methods. Moreover, in our studies the numerical method of calculation of log k_w parameters, basing on linear form of Oscik's equation is applied.

Different indices, log *P*, log k_w and ϕ_o, were proposed and compared as descriptors of hydrophobic properties of 22 of dihydroxythiobenzanilides – organic substances characterized by antifungal properties. The log *P* values were calculated using theoretical methods proposed at http://146.107.217.178/lab/alogps. Chromatographic parameters log k_w and ϕ_o, were calculated from experimental TLC data obtained for RP-18W and RP-CN bounded stationary phases and methanol or acetone applied as organic modifiers. Apart from extrapolation the numeric method was applied for calculation of log k_w values. The effect of mobile and stationary phase properties on chromatographic parameters was analysed and calculated hydrophobicity index were compared with biological activity of test substances.

CHEMOMETRIC ANALYSIS OF RETENTION DATA FROM HPLC – STRUCTURAL PARAMETERS AND HYPOLIPIDEMIC ACTIVITY OF α-ASARONE DERIVATIVES

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The aim of work was researching relationship between biological activity, chromatographic retention parameters and molecular modelling descriptors series of 21 α-asarone derivatives of hypolipidemic activity.

High correlations between the chromatographic retention parameters determined on HPLC system diverse under regard stationary and mobile phases show on possibility uses every of arrangements to valuation lipophilicity.

It was affirmed that there is linear correlation between determined index lipophilicity and calculated lipophilicity (clogP). From here, for individual series of analogs, the parameters are exchangeable. It does not concern the series of α-asarone isomers, that is why the calculated values are equal. However, appointed indices take into account differences in position of methoxy groups in benzene ring.

Computerized methods of multivariable data processing, such as principal components analysis (PCA), which allow the extraction of systematic information from large sets of diverse, and manually interrelated biological, chromatographic and molecular modelling parameters were applied.

For individual series of analogs unexpected and discordant behavior with binding biological activity with lipophilicity theories was observed. It can be bounded with conversion of propenylbenzene derivatives in organism. Mechanism of their biotransformation leads by suitable carbo-cation and alcohol. It was supposed that these transitory forms could show biological activity.

Question with exacting further studies stays the matter of finding uniform and homogeneous scale of biological activity and quantum-mechanical parameters describer the structures of transitory metabolites of α-asarone derivatives.

QUANTITATIVE STRUCTURE-RETENTION RELATIONSHIPS OF STEREODEFINED 2-ALKYLIDENE-4-OXOTHIAZOLIDINES BY RPTLC AND MLR

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Numerous synthetic and naturally occurring 2-alkylidene-4-oxothiazolidines have diverse biological activity [1, 2]. The lipophilicity of the compounds of therapeutic significance is an important physicochemical property. Based on our interest in the biological activity of these compounds, the lipophilic character of 24 functionalized 2-alkylidene-4-oxothiazolidines was studied by means of reversed phase thin layer chromatography, using a mixture of methanol-water as the solvent system. The R_M^0 values were determined for various concentrations of solvent mixtures by linear extrapolation to pure water.

Two types of quantitative structure-retention relationship (QSRR) study were employed to determine lipophilicities of the investigated compounds. The oldest type of QSRR correlates the lipophilicity parameters (R_M^0) with *n*-octanol-water partition coefficients (log*P*). Moderate correlation coefficient was obtained due to the limitations of computational programs giving rise to identical log*P* values for configurational isomers. Second type of QSRR was performed on the lipophilicity parameters in terms of quantum chemical and structural descriptors based on calculation chemistry. The geometries of the molecules were calculated using semiempirical quantum-chemical method AM1 with full geometry optimization, and molecular structures of each configurational isomer were encoded with the large number of descriptors. Multiple linear regression (MLR) analysis was carried out in stepwise manner to select suitable variables and consequently the best model with 4 input descriptors was chosen.

The linear correlation between the predicted and observed values is an indication of the quality of the model assumptions. The proposed model could provide information on the lipophilicity of a potential drug candidate, including useful data for further QSAR investigations.

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USE OF DENSITOMETRY TO DETERMINE OF THE PARTITION COEFFICIENT OF SELECTED ORGANIC COMPOUNDS

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This work is continuation of study, conducted in Chemistry Institute of Silesian University, on the phisicochemical properties of the organic compounds [1-4]. One of the basic phisicochemical parameters in describing both pharmacodynamic and pharmacokinetic aspects of drug action is lipophilicity, which can be expressed by the partition coefficient (log P) of the investigation substances in 1-octanol-water system.

Reversed-phase thin-layer chromatography method and densitometry in the range of the ultraviolet light were used to determine of numerical values of log P selected organic compounds. The subjects of investigation were following substances: acetophenone, aniline, anisole, phenol, and pyridine.

Numerical values of the partition coefficient obtained in this work were compared with literature data and theoretical data.

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ADSORPTION DRIVEN RETENTION IN LIQUID – SOLID CHROMATOGRAPHY WITH TERNARY MOBILE PHASES

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Many works were devoted to the retention in multicomponent mobile phases. They can be divided into two groups. The first class of research focuses on strategies for optimisation separation in systems consisting of three or more solvents. We can mention here the sequential simplex method, the mixture design statistical approach or the Prisma method. These strategies have semi-empirical character, and do not follow from the modern theory of adsorption from multicomponent solutions. The other group contains studies that give insight into molecular mechanism of the separation in complex mixtures. These treatments lead to the analytical expressions for the capacity ratio as a function of the composition of the multicomponent solvent. The composition of the mobile phase is widely used for controlling the separation in liquid chromatography. In this order, retention should be expressed as a function of the mobile phase composition. In the study a series of very simple dependences between parameters characterizing retention in ternary and binary solvents is presented. The linear relationship is proposed to predict the capacity factor in the ternary mobile phase for which the ratio of mole fractions of two less polar solvents remains fixed. Theoretical concepts are verified by comparison of the calculated values with experimental data measured in great concentration region.

The purely adsorption model of retention has been developed for chromatographic systems with ternary mobile phases. The approach leads to equation which permits the calculation of the capacity factors in ternary mobile phases by means of the capacity factors estimated for pure solvents or their binary mixtures. Such a relationship has been successfully used to describe experimental data. The theory allows for a deeper physicochemical interpretation of best-fit parameters and gives the bridge between the retention and adsorption data. The interesting correlations of retention parameters estimated for different mobile phases have been found.

The model permits greater insight into possible mechanism of the process and its evolution with the change of mobile phase composition. It describes chromatographic systems in which the retention is strongly dominated by interactions with the solid surface and the competitive adsorption of all solvents dictates the mode of the process.

The capacity ratio in the ternary mobile phase can be calculated by means of capacity ratios in the binary solvents containing the same mole fraction of the 1st solvent (x_1 = const).

$$\log k_s = \frac{r}{r+1}\log k_{s(1,2)} + \frac{1}{r+1}\log k_{s(1,3)}$$

where k_s, $k_{s(1,2)}$, $k_{s(1,3)}$ is the capacity ratio in ternary (1+2+3) and binary mobile phases (1+2), (1+3) respectively. Parameter $r = x_2/x_3$.

The chromatographic measurements were made using thin-layer adsorption chromatography. The components of mobile phase were: hexane, n-heptane, cyclohexane, ethylene chloride, toluene and acetone. In all mixed mobile phases concentration of the most polar solvent ‘1’ was equal to 0.3, 0.5, 0.7, 0.9 and 1.0 of molar fraction. In the preparation of the ternary mobile phases the concentration of the binary phase (2+3) was first defined i.e. their molar ratio ($r = x_2/x_3$) was equal to 1/3, 1 and 3. Then the most polar solvent was added.

TEMPERATURE – TOOL IN SEPARATION OF ALKALOIDS BY RP-HPLC

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Alkaloids, possessing heterocyclic nitrogen are difficult subject of chromatographic analysis. It is because of dual nature of interactions in chromatographic reversed phase systems – as neutral neutral molecules by hydrophobic interactions with stationary phase ligands and as ions by ion-exchnage interactions with surface residue silanols. It causes widening of peaks and makes separation and determination of alkaloids hardly possible.

To reduce this effect various methods can be applied: the use of buffered aqueous eluent at low and at high pH to supress silanols' or alkaloids' ionisation; the use of anionic ion-pair reagents to form neutral associated, the use of ammonia or amines as silanol blockers.

It causes improvement of peaks' shape – peak symmetry and system efficiency. It causes also optimisation possibilities of systems for the obtainment of satisfacory separation of mixture components.

The change of temperature gives also possibilities of influence of peak shape and separation selectivity especially for ionogenic substances. Because of that we have examined the effect of temperature on the peak symmetry and theoretical plate number obtained for investigated alkaloids in different systems. Various stationary phases such as C18, phenyl and pentafluoro-phenyl and aqueous eluent systems consisting of acetonitrile, buffer at pH 3.5, ion-pair reagents (OSA-Na, PSA-Na) or disthylammine were applied. The most effective systems were: penta-fluorophenyl phase / aqueous mobile phase with addition of DEA. The most visible temperature effect can be observed on phenyl stationary phase. The optimum temperature was found to be 35-40^{0}C in most cases.

The best selectivity of separation of investigated alkaloids was obtained by use of C18 column and aqueous eluent containing DEA or pentafluorophenyl column and aqueous eluent containing DEA.

INFLUENCE OF SAMPLE – APPLICATION MODE ON SEPARATION PERFORMANCE IN PRESSURIZED PLANAR ELECTROCHROMATOGRAPHY SYSTEMS

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Planar electrochromatography (PEC) is the mode in which mobile phase is driven into movement by electroosmotic effect relative to the stationary phase (adsorbent layer of the chromatographic plate). Increasing number of papers in scientific literature has been published on this mode in the last decade. Especially promising results were reported by Nurok et al. [1]. They have described new device for PEC in which adsorbent layer of the chromatographic plate was covered with Teflon foil and ceramic sheet which were pressed to the chromatographic plate. Then the separation system was closed - no vapor phase was present in the system. The authors have named the mode as pressurized planar electrochromatography (PPEC). We designed in the department the chamber for PPEC too and performed experiments which are promising for application in laboratory practice [2].

Many different variables influence separation efficiency in PPEC e.g. electric field strength, potential zeta of the stationary phase - mobile phase interface, viscosity of the mobile phase, types of the stationary and mobile phases, mode of sample application and others. Influence of these variables is under investigation by some groups of researchers. We present our last investigations concerned with influence of sample application mode on performance of the separation system of PPEC. Different modes of sample application such as manual application with hand operated microsyringe, application with automatic applicator, and application using modified technique in the laboratory are compared. The last mode is especially promising due to possibility to perform the application of many samples on one plate and to wet the adsorbent layer with the mobile phase before PPEC experiment. This procedure leads to higher repeatability and efficiency of separations.

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INFLUENCE OF THE BINARY MOBILE PHASE ON CHROMATOGRAPHIC SEPARATION OF PORPHYRINS BY MEANS OF PARTITION THIN LAYER CHROMATOGRAPHY. PART II

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Problem of selection conditions of chromatographic separation of substances brings to choice of compactly definite mobile phases and stationary phases. Large quantity solvents, as well as their modifications through creation of binary or multicomponent mobile phases con-siderable degree widens possibilities of investigation of separation process of substances on thin layer chromatography. Because choice definite of mobile phases have analytical and physico-chemical meaning. From given literature results, that investigation of influence of mobile phases (mono, binary, and multicomponent) on value parameters of substance separation was and is object many of well founded scientific publications. Object of present work are investigations of influence of following binary mobile phases: methanol: chlorinederivative and ethanol:chlorine-derivative on separation of selected porphyrin by means of partition thin layer chromatography.

INVESTIGATION OF THE ASYMMETRY OF MICROCRYSTALLINE SILICA GEL BY MEANS OF THE SPECTROSCOPY OF CIRCULAR DICHROISM (CD)

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Plain silica gel is the most popular adsorbent used for coating of thin-layer chromatographic plates. Moreover, it is widely used as a hard-core matrix used for production of the organic chemically bonded stationary phases with the apolar and polar ligands (like, e.g., RP-2, RP-8, RP-18, CN, NH_2, and Diol). It has always been accepted for granted that silica gel cannot extend any specific impact on chiral analytes and – contrary to, e.g., cellulose powder or paper – it has never been considered as a chiral sorbent.

In our experiments with the selected chiral drugs we managed to demonstrate that the migration tracks of these analytes, when developed on the silica gel layers in the normal chromatographic tanks operating in the ascending mode, show a marked deviation from verticality (5-mm deviation along the 15-cm migration route). These experiments were repeated many times and all the external and purely physical factors could be excluded. Moreover, we used the selected dyes as the non-chiral control solutes and in this case no deviation from strict verticality has been observed.

Our striking results with the deviation from verticality of the chiral analytes' migration tracks allowed us to formulate the working hypothesis concerning chirality of silica gel. The starting point for our considerations was the fact that silicon dioxide (chemically identical with the silica gel used in chromatography) can crystallize as quartz (i.e. the rock crystal) in the two asymmetric – left-handed and right-handed – forms. In order to verify our hypothesis, we run the spectra of circular dichroism (CD) and also the UV-Vis spectra for the samples of the binder-free silica gel used for coating of the commercial chromatographic plates, and separately of the binder itself. As expected, the CD spectrum of the binder did not reveal any measurable Cotton effects, thus eliminating this particular substance from our further considerations. In the case of the investigated silica gel sample devoid of the binder, the two well pronounced Cotton bands – one positive and one negative – were recorded.

The origin of these Cotton bands in the CD spectrum of silica gel seems not very difficult to explain. The silica gel sample can be contaminated with trace amounts of the adsorbed non-chiral organic compounds, having the chromophoric functionalities (e.g., the carbonyl groups). These compounds can be, e.g., the adsorbed organic solvents from an ambient air in the course of the plate storage. Deposited on an asymmetric support, the electron orbitals of the adsorbed molecules are affected by its asymmetry. In fact, these molecules behave as the asymmetric species and in the case of quantitative predominance of one type of the asymmetric microcrystalline variety, the Cotton bands are apt to appear in the CD spectrum of the investigated silica gel.

A STUDY ON THE INFLUENCE OF WATER ON THE RETENTION OF IBUPROFEN AND NAPROXEN IN HPLC WITH THE C-18-TYPE STATIONARY PHASE

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In our earlier studies [1-3] we demonstrated a striking behavior of the selected chiral 2-arylpropionic acids (APAs), when stored for the longer periods of time dissolved in certain solvents. Namely, these compounds show an ability to transenantiomerize in a repeated, i.e. oscillatory, manner, thus repeatedly changing their spatial arrangement from the *S* to the *R* form and *vice versa*.

Oscillatory reactions are not very frequently encountered in the laboratory practise and their occurrence needs fulfilment of certain inevitable preconditions. Among the most popular generators of oscillatory reactions is an increase of viscosity in the reaction environment. Once the rate of formation of an intermediate product in a given multi-step reaction surpasses that of its diffusion, the precondition of an oscillatory reaction is fulfilled. This particular mechanism seems responsible for oscillatory transenantiomerization of chiral 2-arylpropionic acids also.

Oscillatory transenantiomerization of APAs occurs entirely in the spirit of the first oscillatory reaction reported and explained by Zhabotinskii and Byelousov, and it can be given by the following scheme [4]:

S-(+)-2-APA ⇔ keto-enol tautomer ⇔ *R*-(-)-2-APA

Keto-enol tautomerism is known to be efficiently catalyzed in the basic environment and fully hampered in the acidic one. The best pronounced oscillatory transenantiomerization of the selected APAs was observed in our studies for the APA samples dissolved in a mixture of ethanol and water (7:3, v/v). It seems that this binary mixture can easily be considered as an ampholytic solvent, thus able to both catalyze and hamper the tautomerism.

In this study we are going to present the results of our investigations of the gelating properties of *S*-(+)-ibuprofen and *S*-(+)-naproxen as the low-molecular-weight gelators, systematically carried out with use of the HPLC system with the C-18-type stationary phase. We focused our attention on the changing amounts of water contained in the binary solvents of the two investigated APAs and also in the mixed mobile phases, and on the specific impact of water on the chromatographic response. The specificity of such response consists in the highly asymmetrical concentration profiles of the APAs, which seem to well correspond with the state of the high viscosity of the respective APA-containing moieties.

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ON THE DETECTION PROBLEMS WITH THE SELECTED 2-ARYLPROPIONIC ACIDS (APAS) TRACED BY MEANS OF UV DETECTORS

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The most common detection systems both in high performance liquid chromatography (HPLC) and in the instrumental thin layer chromatography (TLC/densitometry) are based on light absorption of the analytes in the UV-Vis region. In the case of the analytes containing one or more aromatic moieties in their molecular structure, the sensitive UV detectors play a leading role in quantification of the analytes. The respective calibration curves are founded on the quantitative UV-Vis absorption of the analytes and the HPLC/UV and TLC/UV analyses of drugs make a considerable part of various national and international pharmacopoeias.

In our recent studies [1-3], we reported on a striking phenomenon of the repeated, i.e. the oscillatory, transenantiomerization of the selected non-steroidal anti-inflammatory drugs (NSAIDs) from the group of 2-arylpropionic acids (APAs). This oscillatory transenantiomerization occurs entirely in the spirit of the first oscillatory reaction reported and explained by Zhabotinskii and Byelousov, and it can be given by the following scheme [4]:

S-(+)-2-APA ⇔ keto-enol tautomer ⇔ *R*-(-)-2-APA

It is obvious that UV spectra of the two antimers, i.e. of the pair of a given *S*-(+) and *R*-(-)-2-arylpropionic acid, are identical (or almost). However, the electron structure of an intermediate keto-enol tautomer is different from that of the original acid and for this reason an assumption can easily be made that UV spectrum of the respective tautomer will also be different. It can further be deduced that – depending on the amount of a given intermediate keto-enol tautomer in the environment – the UV spectrum of a given pair of APAs can be overlapped to a greater or lesser extent by that of the tautomer, thus falsifying the quantification result with an APA in a given sample.

It was the aim of this study to demonstrate the impact of oscillatory transenantiomerization of the selected APAs on the respective UV spectra and the chromatographic UV-absorption-based instrumental detection. From the results obtained it becomes quite clear that – due to oscillatory transenantiomerization – the UV-based quantification of the APAs can be considerably less accurate than it might be expected from the sophisticated detection techniques employed.

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ON THE TWO-DIMENSIONAL SEPARATION OF CHIRAL 2-ARYLPROPIONIC ACIDS (APAS) BY ONE-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY (TLC)

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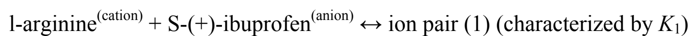
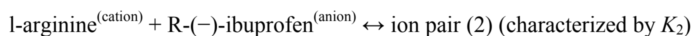
Among the evident advantages of thin-layer chromatography over the other chromatographic techniques, its simplicity and rapidity are the features most frequently appreciated by the analysts. Separation of chiral 2-arylpropionic acids (APAs) is one of the important tasks in the field of separation science, due to the fact that a considerable number of APAs are the non-steroidal anti-inflamatory drugs (NSAIDs). In the course of our investigations on physicochemical properties of ibuprofen, naproxen, and 2-phenylpropionic acid (all three belonging to the APAs), we discovered the most striking ability of L-arginine as chiral selector to provide the two-dimensional (2D) separation of their respective antimers in the one-dimensional TLC systems. Demonstration and discussion of this striking ability makes the subject of this paper.

The starting point for our studies on performance of TLC in chiral separations was the paper by Bhushan and Parshad [1], describing separation of *S*-(+)- and *R*-(-)-ibuprofen on the silica gel layer impregnated with L-arginine as chiral selector. The authors proposed the below given mechanism of the ion-pair formation to explain the separation achieved:


l-arginine$^{(cation)}$ + S-(+)-ibuprofen$^{(anion)}$ ↔ ion pair (1) (characterized by K_1)


l-arginine$^{(cation)}$ + R-(−)-ibuprofen$^{(anion)}$ ↔ ion pair (2) (characterized by K_2)

where ($K_1 \neq K_2$).

We adapted the chromatographic conditions presented in paper [1] to the analysis run on the commercially precoated chromatographic glass plates and to densitometric detection. Besides, we modified quantitative composition of the mobile phase proposed in paper [1] to achieve separation of the naproxen and 2-phenylpropionic acid antimers also. In paper [2], the modified thin-layer chromatographic conditions, suitable for separation of the antimers of each of the three investigated APAs, were introduced.

Densitometric detection of the respective one-dimensional thin-layer chromatograms revealed that – surprisingly enough – L-arginine used as chiral selector provides two-dimensional separation of the three pairs of antipodes (for ibuprofen, naproxen, and 2-phenylpropionic acid), in the direction of the development of the chromatograms and also horizontally, i.e. in the direction perpendicular to the former one. Difference in the migration track lengths with the two antimers is a fully expected effect, but simultaneous bending them to the left and to the right from verticality can be viewed as a surprise bonus and an obvious "stereopeculiarity", which enhances separation and is only possible with planar chromatography. Up to our best knowledge, this is the first report in chromatographic literature on the left-handedness and the right-handedness of the antimers' migration tracks in TLC. The phenomenon certainly has its interesting molecular-level preconditions, which are worth of further scrutiny.

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CHEMICAL STABILITY OF PORPHINE INVESTIGATED BY TLC

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Strong interest in the chemistry of porphyrins has been generated by their numerous applications in various fields of chemistry, biochemistry and medicine.

Hereby is the latest report on progress in porphyrin research carried out at the Institute of Chemistry, University of Silesia in Katowice.

The other problem of the investigation is chemical stability of the presented group of porphyrins and metalloporphyrins. It is generally known that these chemical substances are of low chemical stability and require particular conditions of storage.

Different research carried out so far has shown that densitometric analysis presented changes in the spectrodensitogram of porphine after same time. This caused further investigation into chemical stability of porphine.

The aim of the presented research was to:
- investigate the influence of time on the chemical stability of porphine,
- investigate the influence of H_2O_2 on the chemical stability of porphine,
- investigate the influence of H_2O on the chemical stability of porphine,
- determine the conditions of storage of porphine on thin-layer chromatogram.

NP TLC AND RP TLC FOR QUANTITATIVE DETERMINATION OF IBUPROFEN IN SELECTED PHARMACEUTICAL FORMULATIONS

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Huge demand for painkillers in the modern world forces pharmaceutical companies to produce large amounts of such formulations. It is necessary to control quality of produced pharmaceutical formulations. Detailed control of quality concerns mainly the degree of purity of pharmaceutical formulations. Therefore, the investigations have been undertaken to separate and determine the quantity of ibuprofen in pharmaceutical formulations.

Ibuprofen, [(R,S)-α-methyl-4-(2-methylpropyl)-benzeneacetic acid), is a non-steroidal anti-inflammatory drug (NSAID) used in treatment of pain and inflammation in rheumatic disease and other musculoskeletal disorders. Pharmaceutical formulations of ibuprofen in Poland are produced by many different companies.

The aim of the research was to separate ibuprofen from matrix and quantitative determination of the drug in pharmaceutical formulations using absorption (SiO_2) and partition (RP 18) thin-layer chromatography and densitometry.

THIN-LAYER CHROMATOGRAPHY IN MAGNETIC FIELD

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Chromatography is a very successful method for separation and identification of the some components of the mixtures. However there are a lot of the chromatographic techniques, all time works in order to improve the separation effect of the mixture components are in progress. The works are carried out in two ways: one of them is improve the chromatographic apparatus, searching w new stationary phases (in chemical and physical aspects) and elaboration of a new chromatographic techniques. Separation of the more complicated mixtures, oblige to search of new chromatographic techniques. It is widely know, that the magnetic field influences the substance properties. So we decided to test, how magnetic field influences on the chromatographic process. In order to realize the task a special chromatographic systems with a strong neodymium magnets are constructed. Chromatography process were carried out in strong magnetic field created by neodymium magnets. In the presented investigations the development time, retention parameters and efficiency of the chromatographic systems in magnetic field are compared with the values obtained in classical chromatographic systems (without magnetic field), and some significant differences have been shown. The presence of magnetic field changed the R_f values. The changes dependent on: migration distance of the mobile phase, qualitative and quantitative composition of the mobile phase and structure of the chromatographed solutes. As mobile phase benzene-aliphatic hydrocarbon binary solutes have been used. The kind of the hydrocarbon influence on the changes of the retention parameters of the solutes in magnetic field (for n-hexane stronger effect of electric field has been observed than for n-octane). The magnetic field influences also band/spot width, but for now, it is difficult to find some correlations between parameters of experiment and width of mentioned above width.

Times of development in the magnetic field are generally few percent shorter than without it, and it is very possible that further increasing of intensity of field may lead to better improvements in this branch of investigations.

Described experiments are very promising. Thus it is not a great breakthrough but it is very possible that in the future the combination of chromatographic techniques and magnetic fields will become a new branch of chromatographic sciences.

M-ZSM-5 ZEOLITES (M = Na, H) WITH DIFFERENT SiO_2/Al_2O_3 RATIO AS POSSIBLE NEW STATIONARY PHASES IN PLANAR CHROMATOGRAPHY

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Zeolites are inorganic materials with large surface areas and well-defined internal structures of uniform cages, cavities or channels. They are hydrated aluminosilicates belonging to the family of the tectosilicates, where the SiO_4 tetrahedra form three-dimensional supercages [1]. In their structure some Si atoms are substituted by Al atoms, resulting in a negatively charged structure that originates from the difference between the $(AlO_4)^{5-}$ and $(SiO_4)^{4-}$ tetrahedra [2]. These negative sites are balanced by counterions, usually alkaline and alkalineearth cations.

Unlike the external surface such as that of a conventional high surface area amorphous silica, the atoms of intercrystalline surface is made of atoms and bonds of the bulk structure. Thus, one would expect that the Si-O-Si bonds of this structure should be hydrophobic, on the other hand all commonly known zeolites show strong affinity for water [3].

They are widely used as sorbents and catalysts in variety of processes within chemical, petroleum, petrochemical and food industry [4].

The framework of this study is to examine behavior of ZSM-5 zeolites with different SiO_2/Al_2O_3 ratio as stationary phases in TLC. We used three types of zeolites ZSM-5 structure (SiO_2/Al_2O_3 ratio 42, 50 and 280) with sodium and hydrogen as counterions. The applicability of mentioned materials was checked by separation of a few classes of metal complexes.

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A SEMI-AUTOMATED APPROACH FOR MULTIVARIATE ANALYSIS OF SECOND-ORDER INSTRUMENTS

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Nowadays, we are witnessing a significant increase in the amounts of data produced in a laboratory. As a consequence, an urgent demand for computer-aided analysis is emerging, in order to convert these vast amounts of data to information (and, moreover, to knowledge). In this context, the development of unsupervised methods becomes crucial. When a complex program is developed, asking for user intervention at each step can constitute a great disadvantage, decreasing significantly the usefulness of the entire data-treatment program. In this communication, a statistical method to analyse complex two-dimensional chromatographic data sets ("chroma^{2}grams") is presented. The method is based on the application of the Alternating-Least-Squares [1] procedure using the Orthogonal-Projection Approach [2], not in a single step, but recursively. An unsupervised determination of the number of underlying compounds in a 2D peak cluster is performed on-line, based on the Durbin-Watson criterion [3].

Although the software program is quite sophisticated, our aim is to make it useful to practical chromatographers, without a background in chemometrics. Advantages and drawbacks of the proposed approach are discussed.

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OPTIMISATION OF COMPREHENSIVE TWO-DIMENSIONAL LIQUID CHROMATOGRAPHY SEPARATION SYSTEMS

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In this communication a protocol is proposed for establishing suitable column dimensions (length and diameters), particle sizes, flow rates, and second-dimension injection volumes (i.e. loop sizes) in comprehensive two-dimensional liquid chromatography (LC×LC).

The chromatographer should select the maximum allowable first-dimension retention time, which is approximately equal to the overall analysis time. Also, (s)he should define the maximum allowable pressure in both dimensions and the (minimum) diameter of the first-dimension column. The proposed protocol provides design parameters corresponding to the ideal (theoretically optimal) conditions or to realistic practical conditions. The protocol also allowed us to study the implications of contemporary developments in LC, such as the use of high temperatures (implying reduced viscosities and increased diffusion coefficients), monolithic columns (implying smaller flow-resistance factors), and ultra-high-pressure LC.

The combination of (reversed-phase or normal-phase) liquid chromatography with size-exclusion chromatography (LC×SEC) is frequently employed for analysing complex polymers. The proposed protocol is used to design a suitable LC×SEC system under realistic conditions. The results resemble the systems that have been designed and implemented by expert chromatographers, but they also indicate how current practice can be improved.

MODELING IEC AND v-IEC OF SELECTED ACIDS WITH A MODIFIER ON STRONGLY ACIDIC RESIN IN THE H^+ FORM

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Ion Exclusion Chromatography (IEC) finds many applications in various analytical separations of weak acids. Pure deionized water or a diluted, aqueous solution of a strong acid (such as e.g. sulphuric acid) is used as mobile phase, whereas typical stationary phase is a strongly acidic resin in the H^+ form (e.g. sulfonated polystyrene divinylbenzene (PS-DVB) resin of high ion-exchange capacity, provided by sulfonic acid groups). When pure water is used as a mobile phase, then characteristic leading (i.e. frontally tailing) peaks are obtained and the retention depends mainly on the concentration of the analyte. An alternative technique is the vacancy Ion Exclusion Chromatography (v-IEC) where the column is equilibrated with the sample solution flowing as mobile phase through the system, whereas pure water is injected as a sample. In this case, characteristic negative "vacant" peaks are obtained. Although in v-IEC the main retention mechanism (based on the exclusion of ions) also can be coupled with a complementary adsorption of an analyte on the resin phase the resulting vacant peaks are much more symmetrical.

Peak fronting results from an obvious lack of the buffer capacity with an aqueous eluent and for this reason the pH of the eluent should be maintained constant. Since an increased proton concentration suppresses ionization of the sample acids, a small addition of a strong mineral acid allows for high performance separations by an improvement of the peak shape and of detection sensitivity. Unfortunately, the suppression of the acids dissociation enhance a strongly hydrophobic interaction between the acids and the surface of the stationary phase, resulting in extremely long retention times and strongly tailed peaks obtained for hydrophobic acids, such as aromatic and higher aliphatic carboxylic acids.

The methods employed in order to avoid the peak tailing in IEC include a modification of the mobile phase properties, which is achieved by adding minor quantities of a modifier – in this case heptanol. Higher alcohols are reported to be the most effective organic modifiers in IEC of hydrophobic acids. Application of acidic eluent containing higher alcohol in a very low concentration considerably reduces the retention time and improves the peak shapes. Heptanol seems to be the most efficient modifier because of the limited solubility of the higher alcohols.

The aim of the present work is to describe the retention mechanism in IEC and v-IEC for the adsorptive acids in analytical and concentration overload conditions, using three different mobile phases, i.e. pure, deionized water, diluted sulphuric acid solution and the acid solution with a small addition of heptanol (C7 alcohol). To attain the goal, the Craig model coupled with the equilibrium relationship between the acids, their ions and alcohol, implemented with an appropriate isotherm model, was employed.

The retention times and peak shapes predicted by the derived equations remain in a good qualitative and quantitative agreement with the experimental data.

NEW CRITERION FOR GC-MS IDENTIFICATION OF FLUORINATED ORGANIC COMPOUNDS

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The presence of fluorine in the molecules of organic compounds often cannot be revealed using standard low resolution mass spectra owing to the existence of single stable isotope of this element (^{19}F). The same problem exists for compounds containing mono-isotopic iodine (I). To solve this problem the joint interpretation of mass spectra and GC retention indices (RI) is required.

Integer mass numbers both parent and daughter ions can be presented not only in the standard decimal form, but in numerical system with the basis 14 (the mass number of homologous difference CH_2) [1]:

$$m/z \equiv y(\text{mod}14), \quad \text{or} \quad m/z = 14x + y, \quad x = \text{int}[(m/z)/14]$$

Parameter x for parent ions (if registered) can be compared with values of any additive properties (A) of organic compounds in the form of so-called homologous increments:

$$i_A = A - x\Delta A(CH_2),$$

where $\Delta A(CH_2)$ – the increment of A-values for homologous difference.

If the additive property A is chromatographic retention indices, $\Delta RI(CH_2) \approx 100$, hence

$$i_{RI} = RI - 100x.$$

If analytes contain only one F-atom in the molecules, it is impossible to distinguish them unambiguously from non-fluorinated compounds using this criterion. However, if the number of F-atoms in the molecule is three or more, it leads to large negative i_{RI} values, when the number of interfering compounds with the same i_{RI} values seems relatively small (only high branched structures). Hence, the following criterion for revealing not less then three fluorine atoms in the molecules can be proposed:

$$i_{RI} < -120.$$

This criterion is valid only if RIs are measured using standard non-polar polydimethyl siloxane stationary phases. This value evaluated first time and can be defined more precisely afterwards.

Numerous examples confirm this rule, namely: 1,3,5-trifluorobenzene (M = 132, RI = 614, i_{RI} = -286), benzotrifluoride (M = 146, RI = 700, i_{RI} = -300), pentafluorophenol (M = 184, RI = 977, i_{RI} = -323), N-phenyl trifluoroacetamide (M = 189, RI = 1074, i_{RI} = -226), etc.

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MONOLITHIC SORBENTS ON THE BASIS OF HIGH POROUS CERAMIC METERIALS

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Among the existing methods of preparing porous permeable monolithic ceramic materials the methods, providing high permeable porosity, narrow distribution of pore size and required shape of pores have been chosen. However, the application of such materials as sorbents is possible provided developed sorption surface is present. It can be achieved particularly by application of porous silica coatings on their surface. The main characteristics of the obtained materials depend on the whole series of factors, and the most important are nature and composition of the applying mixture for coating, conditions of drying and calcination. Coating of the monolithic ceramic materials can be done particularly by sol-gel method, using silica sols as a source of siliceous material. Porous coatings are formed as a result of successive processes, involving applying of silica sol on a ceramic support, its gelation and further thermal treatment. The process of thermal treatment must provide:

- completion of all stages of silica layer formation (removal of traces of water present in any form, decomposition of the stabilizer, burning-out of the organic substances),
- ultimate formation of coating of the required structure,
- good adhesion of the silica coating to the support.

Various ceramic materials with 80-90% total porosity have been used as starting porous permeable ceramics. The majority of ceramic materials, which were used, possessed rather big pore space at nearly zero specific sorption pore volume ($V_s < 0,1$ cm^3/g). The pore space was represented mainly by macropores. For silica coating, silica-sols, containing particles of different size, have been used. Taking into consideration high porosity of the materials used, the coating has been performed by impregnation method followed by further drying and thermal treatment. Coating by pumping silica sols through pores of the ceramic support has also been used. Conditions of thermal treatment of the applied silica layers have been found by studying processes taking place at increased temperatures. Porous structure of the obtained ceramic materials with silica coating was evaluated by specific sorption pore volume, specific surface and permeability of the sorbent.

The conditions of preparing of monolithic sorbents with given characteristics of porous structure have been found out.

POLYPHENOLIC ACID CONTENT IN FRUITS DURING THEIR RIPENING PROCESS

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Fruit polyphenols are a complex group of aromatic substances presented in more than 4000 different types in plants that have gained enormous attention in the last years, because of their biological and health properties. They are produced in plant as secondary metabolites in shicimic acid biosynthesis pathway. They have been demonstrated to acts as natural antioxidant and assumed to contribute to be beneficial health effects such as anti-inflammatory, anti-histaminic and anti-tumor activities, protection against cardiovascular diseases and as radical scavengers.

The aim of this study was determination of hydroxybenzoic acids (gallic acid, p-hydroxybenzoic and syringic acid) and hydroxycinnaminic acid (ferulic acid, p-coumaric acid, chlorogenic acid, syringic acid) in fruit samples.

Reversed-phase HPLC procedure was optimized for simultaneous determination of both classes of polyphenolic acids. These compounds are the week acids with pK_a from 2,6 to 4,5 so the eluent pH should be below 2,6 because in this pH all compounds exist in undissociated form and could be separate in C-18 column. The most popular pH modifier for such separation are acetic buffer, formic buffer and phosphoric buffer. Only phosphoric buffer has sufficiency low pH for assurance good condition for separation. Mobile phase pH and concentration of acetonitrile or methanol were tested to obtain the best separation conditions. Satisfactory separation was achieved in gradient mode with a mobile phase consisting of 50 mM phosphate buffer with pH=2.2 (solvent A) and acetonitryle (solvent B). The flow rate was kept at 1 mL/min. LOD values were in the range of 0,2-1,0 µg/ml respectively using spectro-photometric detection at 280 nm. The developed method was applied for monitoring the concentration of polyphenolic acids in strawberries, plum and tomato fruits in their ripening process.

DETECTION OF VOLATILE N-NITROSAMINES IN DIFFERENTLY HEATED MEAT PRODUCTS BY GAS CHROMATOGRAPHY – THERMAL ENERGY ANALYZER (GC-TEA)

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The formation and occurrence of volatile *N*-nitrosamines were many times investigated. Most of these *N*-nitroso compounds are potent carcinogenic and might cause tumour in many organs including the kidney, lung, bladder, liver, tongue, and esophagus, depending on the species but not in the brain, bone, colon and skin. They are present in detergents, water, beer, rubber, cosmetics, and various foodstuffs such as cheese, vegetables, mushrooms, milk and meat products. These compounds are formed out of decomposition products of proteins, especially biogenic amines and a nitrosating agent (NO^+) derived from nitrite or nitrate. The most commonly occurring volatile *N*-nitrosamines in meat products are NDMA (*N*-nitrosodimethylamine) and NDEA (*N*- nitrosodiethylamine). Moreover *N*-nitrosopyrrolidine (NPYR) has been mainly detected in fried bacon. Other *N*-nitrosamines were found occasionally such as *N*-nitrosopiperidine (NPIP) and *N*-nitrosomorpholine (NMOR). A great part of work has been done in studying the relationship between acute toxicity and structure of these compounds. It has been proved that acute toxicity decreases with the chain length of dialkylnitrosamines. There are several factors influence the *N*-nitrosamines formation in meat products such as: pH, presence of oxygen, nitrate or nitrite, spices and herbs. The presence can be caused by smoking conditions but also the method of cooking, and frying temperature and time, what was the main goal of these studies. Some authors suggested that among cured meat products bacon is generally nitrosamines free in the raw form and nitrosamines are formed only during the high-heat frying. It was observed that similar high-heat cooking of other cured meat products might lead to the formation of increasing levels of nitrosamines for example: NDMA, NPIP and NPYR in some spiced meat products and hams.

Volatile compounds given above were vacuum distilled in alkaline environment and extracted with dichloromethane. Afterwards the extract was evaporated and injected in the GC. The different components were determined by gas chromatography – thermal energy analyzer.

THE ANALYSIS OF FATTY ACIDS FROM SPECIMENS OF RUMINANTS ORIGIN USING CAPILLARY COLUMN GAS CHROMATOGRAPHY

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Introduction: Meat and milk products derived from ruminants are important source of nutrients in human diets, providing high quality protein, essential minerals, vitamins and certain unsaturated fatty acids possessing beneficial health effects. Paradoxically, ruminant fats are relatively more sa-turated than plant oils. Hence, the aim of current study was to determine if improved gas-chromato-graphy methods is able to satisfactory fractionation of saturated fatty acids (from C8:0 to C24:0), octadecanoic acid isomers (*cis/trans*), a mixture of CLA isomers [1], long-chain PUFA and other ca. 400 fatty acids (FAs) in milk, blood plasma, meat and liver derived form cows and sheep.

Experimental: For the development of the chromatographic procedure for fresh animal tissues, 0.2g liver or muscle sample in 0.4ml methanol was homogenized. Next to the homogenate 1ml water, 1.5ml methanol and 1ml chloroform (with the internal standard, i.e. C17:0 or C19:0) were added. The resulting solution was shaken for 1min and then 1ml water and 2ml chloroform were added again. The content was shaken for 1min, centrifuged for 5min at 3000rpm. Finally, the chloroform phase was used for methylation. For FAs assay in milk or blood plasma, 0.4-1ml milk or plasma sample was added to 2ml methanol and 1ml chloroform with the internal standard. The obtained mixture was shaken for 1min and then vortexed again for 1min with 1ml water. To the resulting solution 2ml chloroform was added, shaken for 1min, and finally centrifuged for 10min at 3000rpm. The chloroform phase was used for methylation.

The mild evaporation under Ar at room temperature was employed for lipids chloroform extracts. FA esters (FAMEs) were prepared by methylating under Ar; NaOH/me-thanol (for 10min at 100°C) and then HCl/methanol (for 45min at 100°C) derivatization was used for preparing FAMEs. Then FAMEs were extracted with 5 ml of hexane in the presence of 4ml saturated NaCl solution. FAMEs were fractionated using long capillary column (109m x 0.25mm x 0.2µm film thickness; Varian) for gas-liquid chromatography (GLC) equipped with an FID.

Results: All standards of saturated FAs, octadecanoic acid isomers, a mixture of CLA isomers and long-chain PUFA were separated using the optimized column temperature gradient program. Thus, the new temperature program was as follows: 70°C for 4min; 12°C/min to 150°C then hold for 6min; 8°C/min to 168°C then hold for 27min; 0.75^{o}C/min to 190°C then hold for 10min; 1.8^{o}C/min to 210°C then hold for 15min; 6°C/min to 234°C then hold for 4min; 6°C/min to 236°C hold for 20min; the post-run for 7min at 250°C. The carrier gas was He and the column was opera-ted at constant pressure (211.2 kPa) with the flow rate of 1ml/min. Injector and detector temperature was maintained at 250 and 255°C, respectively. Under the chosen chromatographic condition, we reported the successful separation of all used FAME

standards on a commercially available 109m capillary column. Satisfactory fractionation of monoenoic acids (from C14:1 to C24:1), saturated FAs, *cis,trans*, *trans,trans* and *cis,cis* group of CLA isomers, as well as medium and long-chain PUFA in assayed biological samples was obtained at run time of 130min. The conjugated double-bond geometry and position as well as the purity of these CLA isomers in standards and in plasma, milk, liver and muscle sample was confirmed by GLC-MS and silver-ion HPLC with UV detection (Ag^+-HPLC-UV). Moreover, the combination of Ag^+-HPLC-UV *pre*-fractionation, *pre*-concentra-tion and injection of large amount of a processed sample onto a 109m capillary GLC column appears to be the best analytical tool for more accurate and selective identification of metabolites of CLA isomers (i.e. conjugated C18:3, C20:3 and C20:4) in the liver.

Conclusion: 109m capillary GLA column and the proposed column temperature program appear to be the best compromise for satisfactory fractionation of physiologically important FAs and dura-tion of GCL analysis. Excellent confirmation of the presence of conjugated FAs was obtained using Ag+-HPLC since UV detection distinguish only FAs containing conjugated double bonds, while other FAs are undetectable.

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Py/GC/MS ANALYSIS OF 5-(4'-CARBOXYPHENYL)--10,15,20-TRI(4'-METHYLPHENYL)PORPHYRIN IN THE PRESENCE OF BSA REAGENT

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In our earlier studies [1,2], the pyrolysis - gas chromatography – mass spectrometry (Py/GC/MS) technique was applied for the identification of the pyrolysis products of 5,10,15,20-tetraphenyl-21H,23H-porphine (TPP) and some its derivatives having the same substituents in the para positions of the aromatic rings, such as: $-CH_3$, -OH, $-OCH_3$, $-NHCOCH_3$ and -COOH. The thermal decomposition products of these compounds were obtained during 5 sec. at the temperature of 770 °C inside the Curie point pyrolyser directly attached to the split/splitless injector of the GC/MS instrument. The identification of these products was based on the interpretation of their low resolution mass spectra. In this way, it was found that during the pyrolysis of TPP, it forms more pyrolitic products in comparison with its other derivatives [1,2}.

The aim of this work was the application of the Py/GC/MS method for the analysis of 5-(4'-carboxyphenyl)-10,15,20-tris(4-methylphenyl)porphyrin (CTTP) in the presence of the bis(trimethylsilyl)acetamide (BSA) reagent. In general, this compound maybe used to the further syntheses of porphyrins, especially useful for the biological and medical applications.

It was found that the main pyrolysis products of these compounds are easier to identify by the interpretation of their mass spectra. In this case, the most characteristic pyrolysis products are: pyrrole, benzene, toluene, xylenes, benzoic acid, mono- and disubstituted pyrrole derivatives such as 2-(4'-methyl-benzyl)pyrrole,2-(4'-carboxybenzyl)pyrrole and 2,5-di(4'-methylbenzyl)pyrrole. The knowledge about the chemical structures of these products permits us to get additional information about their pyrolysate composition and also it confirms the results of Py/GC/MS analysis of CTTP.

The obtained results of the application of Py/GC/MS technique in the analysis of CTTP also maybe used for the determination of the chemical structures of similar porphyrin derivatives with unknown structures.

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FREE RADICALS, TOTAL ANTIOXIDANT POTENTIAL AND THEIR ANALYSIS USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Free radicals adversely modify biologically active molecules as well as whole cells and are implicated in various degenerative diseases and aging. Their mediated process have been implicated in the pathogenesis of several diseases. It is widely believed that these modifications are preventable by exogenous antioxidants. There is a need for a method to assess and compare strength of particular antioxidants in order to select these of the highest potential for further development as drugs. However, it turned out that frequently more information (e.g. synergetic effects) is obtained measuring total antioxidant potential (TAP) of biological samples than concentration of particular antioxidants separately.

Results are presented concerning the application of RP-HPLC to the estimation of TAP after hydroxyl radicals generation in the Fenton reaction and their spin trapping with hydroxybenzoate. Samples were monitored using direct current amperometric detector with the glassy carbon electrode, worked at the potential 0.8 V *vs* Ag/AgCl electrode.

The method was applied to the determination of TAP of some types of compounds (biogenic polyamines, catecholamines and polyphenols), herbs and blood serum. The results were compared with those obtained using photometric TAP method based on peroxyl radicals generation by thermal decomposition of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) in the presence of assayed sample and detection with 6-carboxy-2',7'-dichlorodihydrofluorescin diacetate. It turned out that relative value of the antioxidant potential strongly depended on the generated radical.

DETERMINATION OF VOLATILE *N*-NITROSAMINES IN MEAT PRODUCTS WITH SPICES FROM THE BELGIAN MARKETS BY GAS CHROMATOGRAPHY COUPLED TO A THERMAL ENERGY ANALYZER (GC-TEA)

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Volatile *N*-nitrosamines belong to the the group of *N*-nitroso compounds and most of them are very potent carcinogens. They occur in many of products like tobacco, rubber, cosmetics and in a great number of food products for example in meat.

The formation of *N*-nitrosamines can be caused by many factors such as preservatives, spices and herbs addition or contact with elastic rubber nettings. They are formed in results of nitrosation, that is reaction of *N*-nitroso compound with nitrosating agent. The precursor of nitrosating agent is usually nitrite (NO_2^-), which is used as preservative in meat products.

N-nitrosamines can cause cancer in many organs like liver, lung and kidney. The most frequently occurring and dangerous of these compounds are *N*-nitrosodimethylamine and *N*-nitrosodiethylamine. The relationship between structures and acute toxicity shows that toxicity decreases with chain length of *N*-nitrosodialkylamines but cyclic *N*-nitrosamines are also acutely toxic [1].

There were analyzed some tens of meat products with spices on the presence of *N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitrosodibutylamine, *N*-nitrosopiperidine and *N*-nitrosopyrrolidine. The method of sample preparation was low-temperature vacuum distillation (LTVD). The method of determination was Gas Chromatography coupled to a Thermal Energy Analyzer (GC-TEA) with *N*-nitrosodipropylamine as an internal standard. Thermal Energy Analyzer detection, the only detection method that is recognized as specific for volatile *N*-nitrosamines because of detecting these compounds in meat even at the low trace level, is based on the chemiluminescence generated by the decay of the NO_2 group when it is electronically exited [2].

Received results indicate that the addition of spices to meat has an influence on growth of content of *N*-nitrosamines in meat products.

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USING OF POLYESTERACRYLATES FOR PREPARING THE ORGANO-CERAMIC COMPOSITE SORBENTS

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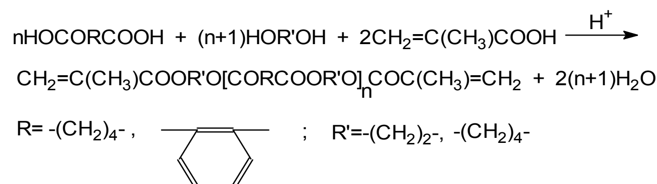
Olygomers with reactive terminal groups capable of setting with formation of polymeric network have certain advantages over monomers. By targeted synthesis of these compounds, changing the nature of the olygomeric fragment as well as reactive terminal groups, changing the length of the network's linkage it is possible to regulate the properties of the resultant polymers. Olygoesteracrylates have been prepared by condensation telomerization, using methacrylic acid as a chain terminator, dicarboxylic acids or anhydrides and diols as polycondensation monomers.



The solution of olygoesteracrylate in benzene or toluene was applied on surface of the ceramic sorbents (either in form of monolith or powder) and was polymerized in the presence of radical initiators. Varying the nature and proportion of bifunctional monomers, olygoesters with different length (degree of polycondensation n) of the olygomeric chain have been synthesized, which allowed to adjust such properties of the resultant polymers as flexibility, adhesion to the substrate surface, porosity of the finished composite sorbent as well as to regulate the balance of hydrophobic and hydrophilic parts of the polymer for selective sorption. The studies, which have been carried out, showed that characteristics of the porous structure (permeability, sorbtion capacity of pores, specific surface) of the initial porous ceramic materials could be significantly improved by applying the polyesteracrylate layers on their surface. The resultant new composite sorbents showed excellent chromatographic performance for rapid separation of both small and large molecules such as proteins and nucleic acids.

APPLICATION OF THE GC-NPD METHOD FOR THE ANALYSIS OF HETEROCYCLIC AROMATIC AMINES

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Heterocyclic aromatic amines (aminoazaarenes) are mutagenic/cancerogenic compounds formed as products of Maillard reaction in many kinds of thermally treated high proteinaceous food. Althought the concentration of them in food samples is low (ppb to ppt) different epidemiological studies among people have indicated a positive relation between the consumption of broiled meat or fish and the increased cancer incidences. These biological active heterocyclic amines were also determined in the investigated 10 meat dishes prepared according to polish and silesian recipes.

In recent years HPLC method with various detection systems has been elaborated for the qualitative and quantitative analysis of aminoazaarenes. The use of gas chromatography coupled with MS or NPD requires the derivatisation of aminoazaarenes to compounds of reduced polarity and increased volatility.

Nitrogen-phosphorous detector NPD is highly-sensitive for organic nitrogen compounds, so the aim of our study was the application of GC/NPD for the analysis of five nitrogen heterocyclic amines derivatised to pentafluoropropyl amides and to N-methylaminomethylene compounds. These derivatisation methods were successfuly applied by us for the identification of aminoazaarenes in meat sample by use of GC-MS.

The investigated aminoazaarenes mixture contained: 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ), 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (4,8-DiMeIQ$_x$), 2-amino-1-methyl-6-phenyl-imidazo[4,5-b]pyridine (PhIP). The used gas chromatograph (GC2010-Shi-madzu) was equipped with a HP Ultra 2 fused-silica capillary column 25 m x 0.2 mm (film thickness 0.25 μm).

DETERMINATION OF PROCANCEROGENIC HETEROCYCLIC AMINES (QUINOLINE, QUINOXALINE AND PYRIDINE DERIVATIVES) BY PLANAR CHROMATOGRAPHY WITH DENSITOMETRY

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Heterocyclic aromatic amines (aminoazaarenes) belong to compounds which may be synthesized from amino acids, creatine and carbohydrates in heat-processed proteinoceous food. There are some types of such amines usually formed in cooked foods, among which the derivatives of aminoimidazoquinoline, aminoimidazoquinoxaline and aminoimidazopyridine are recognized as the highly mutagenic and carcinogenic.
HPLC/MS, HPLC/DAD and GC-MS techniques are the most often applied for analysis of aminoaazarenes in food samples.

The various separation options of five heterocyclic amines such as: 2-amino-3-methylimidazoquinoline (IQ); 2-amino-3,4-dimethylimidazoquinoline (MeIQ); 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (DiMeIQx); 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx); and 2-amino-1-methyl–6-phenylimidazo[4,5-b]pyridine (PhIP) by planar chromatography were investigated using silica gel and RP-18 as stationary phases; as the mobile phase acetonitrile, chloroform, methanol, dichloromethane, diethyl ether and water as well as their mixtures in various composition were applied.

The detection and determination limits for each of the investigated aminoazaarenes were determined at the optimal wavelength in reflection and fluorescence mode by the use of a Shimadzu (Japan) densitometer. It was stated that it was possible to determine methyl deri-vatives of aminoquinolines and aminoquinoxalines as well as of aminopyridine in nanograms range per spot. For improving of the shape of aminoazaarenes spots triethylamine (TEA) or/and sodium 1-octanesulfonate were added to the mobile phase. At the selected TLC conditions (RP-18/acetonitrile-water 88+12 v/v with 0.1% TEA and 40 mg% of octanesulfonate) the separation of quinoline-type compounds from quinoxaline-type ones and PhIP has been achieved.

THE TLC SEPARATION OF HETEROPENTACENES

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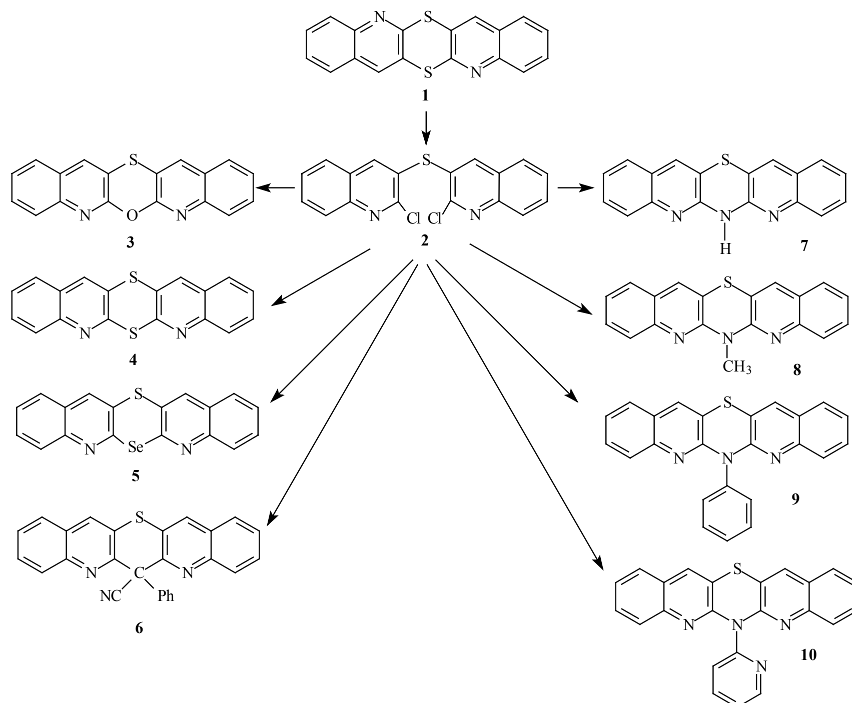
The dithiin ring opening reaction in heteropentacene 1 with sodium methanethiolate and further transformations led to sulfide 2, which with various reagents underwent the ring closure reactions to give heteropentacenes 3 – 10 [1, 2]. The structures of heteropentacenes were determined using spectroscopic data and were confirmed by X-ray analysis [1 - 3].



The progress of these reactions was followed by the TLC analysis. It was important to find appropriate chromatographic conditions enabling separation of the product from the starting material and some by-products. The efficiency of these separations was determined using the retention (R_F, ΔR_F) and separation (α) factors.

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HPLC CHARACTERIZATION OF THE REACTION PRODUCTS OF FORMATION OF SELECTED PYRIDYLPORPHYRINS

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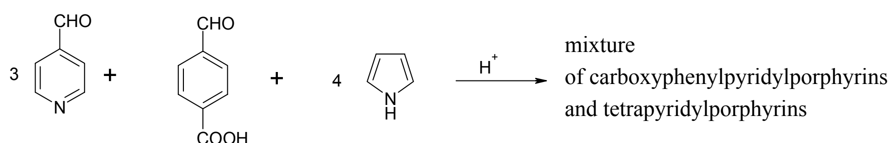
Porphyrins have attracted attention for many decades and are well-known as biological materials (chlorophyll, hemoglobin). Also of their versatile applications such as light-emitting diodes, molecular thermometers, magnets, photosensitizers in photodynamic therapy, dehalogenation agents and optical limiting devices [1,2]. The pyridylporphyrins are class of compounds which may be easily converted into soluble in water porphyrins. It is important in model investigation naturally occurring processes such photosynthesis, breathing in plant and animal world.

Since the first synthesis of a tetraarylporphyrin, in 1935 by Rothemund, substantial improvements in the chemical synthesis were obtained by Adler [3] et al. and more recently by Lindsey et al. [4].

Carboxyphenylpyridylporphyrins were synthesized by the conventional method of Adler using 4-pyridinecarboxaldehyde, 4-carboxybenzaldehyde and pyrrole in the boiling propionic acid.



TLC chromatography has shown six spots which are corresponded to six possible products mentioned above and R_F values were calculated to each spot. The reactions mixture was investigated by HPLC to determine quantitative yield of formed products.

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FRACTIONATION AND IDENTIFICATION OF UNDERIVATIZED POSITIONAL AND GEOMETRIC ISOMERS OF CONJUGATED LINOLEIC ACID

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Introduction: Conjugated linoleic acid (CLA) isomers are physiologically highly active compounds that differ in their biological properties in several ways from linoleic acid. Considering the growing demands for investigation of physiological properties of individual isoforms of CLA and their metabolites, new strategies can be tried to improve determination of conjugated fatty acids (CFAs) from specimens of animal and food product origin. The aim of the study was developing the more versatile liquid chromatographic (HPLC) method with photodiode detection (DAD) for satisfactory detection and distinguishing of *trans,trans, cis,cis* and *cis,trans/trans,cis* CLA isomers.

Experimental: *Freeze dried and powdered rats' liver or muscle samples (50 mg) was treated with 2 ml of 2M KOH in water, 2 ml 1M KOH in methanol, then flushed with stream of Ar. The resulting mixture in a closed vial was then vigorous mixed and heated under Ar at 95°C for 10 min, next cooled for 10 min at the room temperature and sonicated for 10 min.*

Extraction of free fatty acids (FAs): To the hydrolysates in vials 3 ml of water were added and the resulting solution was then mixed. The obtained solution was acidified with 4M HCl to pH ~2 and then free FAs were extracted 4-times with 3 ml of dichloromethane (DCM). The DCM layer was dried with Na_2SO_4. Free FAs extraction was repeated using 4 times 3 ml portion of heptane. Afterward, the heptane layer was combined with DCM layer and then solvents were removed under Ar. Finally, the residue was re-dissolved in 1 ml of DCM and then 20-40 µl of the resulting solution were injected onto the Ag^{+}-impregnated columns.

Ag^{+}-ion chromatography (Ag^{+}-HPLC): Fractionations of free CFA were carried out using isocratic chromatography (two 250x4.6 mm Chrompack ChromSpher 5 µm Lipids columns) and the photodiode array detector (DAD) operated in the UV range from 195 to 360 nm. Runs were performed at a flow-rate of 1.35 ml/min at the column temperature of 25°C. The eluent was prepared from heptane, acetic acid, acetonitrile (ACN) (99.483:0.5:0.017,v/v/v).

Results: Our current chromatographic method allows better fractionation of geometric and positional CLA isomers and other CFA compared with our previous gas chromatography and reversed-phase C_{18}-HPLC methods. Very low background fluctuation was observed and all peaks were absent from the blank, when the current chromatographic procedure was applied. Proposed isocratic elution system composed of acetic acid and ACN in heptane and detection in at 234 nm were found to provide excellent baseline stability. What is particularly puzzling is that heptane only with the higher amount of ACN ensured very small baseline noise and substantial retention of all assayed CLA isomers compared with the amount of ACN in the mobile phase containing hexane. The addition of the greater amount of ACN (from .017 to .02 %) to heptane permitted excellent peak shapes without "tailing", close to symmetrical even with elution times from 25 to 60 min, hence, excellent resolution of all CLA isomer peaks was

obtained. The absorption UV spectra of *cis,cis* and *trans,trans, cis,trans/trans,cis* CLA isomers bear a close resemblance, however, the absorbance maximum evidently depended upon the geometric form of assayed isomers (at 235.4, 231.9 and 234.3 nm, respectively).

Conclusion: Direct injection of mildly saponified lipids to free FAs is to be the most accurate chromatographic method of quantifying CLA isomers and other CFAs. Therefore, direct high resolution Ag^+-chromatography based on DAD should helps in understanding the mechanism responsible for diverse physiological functions reported for CLA isomers and their metabolites, due to more confidential measurement of all CFAs. Satisfactory accuracy, precision and sensitivity (the limit detection: 0.1–1 ng/l) can be achieved for determination of CFAs in blood plasma, muscles or liver of rats or sheep.

EFFECT OF BUTYLATED HYDROXYTOLUENE ON COMPOSITION OF METHYLATED FATTY ACIDS ASSAYED BY GAS CHROMATOGRAPHY

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Introduction: Recently, we have demonstrated that capillary gas liquid chromatography (GLC) and silver-liquid chromatography (Ag^+-HPLC) may be adapted to analyze a mixture of fatty acids containing conjugated double bonds (eg. CLA isomers) as well as other unsaturated and saturated fatty acids. The reproducibility of fatty acids (FAs) assay was satisfactory sensi-tive to small changes in temperature of processing samples, presence of endogenous species biological materials and the content of prooxidative species as well as antioxidant. The concentration of pro-oxidative and anti-oxidant species resulted in significantly affected the profile of particularly mono- and poly-unsaturated FAs (MUFA, PUFA). Thus for optimal quantification, the selection of FAs protection method against oxidation is crucial to success-fully quantifing saturated FAs, MUFA and PUFA, particularly CLA isomers. In current study, two methods have been compared which involve the use of butylated hydroxy-toluene (BHT) (Method BHT) or Ar (Method Ar) to remove of air from processing samples.

Methods Ar and BHT: The first step procedure was mild saponification of a CLA isomer mixture, unsaturated FA methyl esters (FA-MEs) and a lyophilized liver of rats. Assayed samples were treated with a mixture of 2 ml of 2 M KOH in water, 2 ml 1 M KOH in methanol flushed with a stream of Ar (Method Ar) or treated 50 µl of BHT solution in methanol (20 mg/ml) and next flushed with a stream of Ar (Method BHT). The resulting mixture in a tightly closed vial was then vigorously mixed and heated at 95°C for 10 min, next cooled for 10 min at the room temperature and then sonicated for 10 min.

Extraction of free FAs: To the hydrolysate 3 ml of water were added and the resulting solution was than vigorously mixed. The obtained solution was acidified with 4 M HCl to pH 1-2 and then free FAs were extracted four times with 3 ml of dichloromethane (DCM). The lower DCM layer was dried with about 100 mg of Na_2SO_4. To avoid any loss of free FAs extraction was repeated using 4 times 3 ml of *n*-hexane. Afterward, the upper *n*-hexane layer was combined with DCM layer and then organic solvents were removed under a stream of Ar.

Preparation of FA-MEs: To the residue was added 2 ml of 2 M NaOH in methanol, and next flushed with a stream of Ar (Method Ar) or treated with 50 µl of BHT solution in methanol (20 mg/ml), and then flushed with Ar (Method BHT), and next reacted for 1 h at 80°C. After cooling to reaction mixture 2 ml of 25% BF_3 in methanol were added, flushed with Ar for 3 min (Method BHT) or for 5 min (Method Ar), and again heated for 1 h at 80°C. To a cooled reaction mixture 5 ml of water was added and then FA-MEs were extracted with 5 ml of n-hexane. The supernatant was transferred to a vial. Separation of all FA-MEs was carried out using GLC, while FAs containing conjugated double bonds using also isocratic liquid chromatography (Ag^+-HPLC) with photodiode array detection (DAD) at 234 nm.

Verification: The obtained results show the difference in the profiles of MUFA and PUFA present in the FAs-standard mixture as well as in assayed liver. Method Ar was more associated with increase the peak areas of all CLA isomers in comparison with ones obtained from analysis performed according to Method BHT.

Conclusions: The current study provides the universal method for gentle saponification and next the base-acid catalyzed methylation of FAs in obtained hydrolysates. The results show that the saponification and derivatization in the presence of BHT (Method BHT) is to be the more accurate method for quantifying FAs in a commercial standards and biological samples. However, the influence of BHA as well as other anti-oxidants on the accuracy of FAs assays should be further studied.

THE RATE OF VAPORISATION OF CYCLOHEXANE IN A CONCENTRATOR AND RECOVERY OF POLYCYCLIC AROMATIC HYDROCARBONS (PAH)

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Analysing e.g. plant materials for traces of polycyclic aromatic hydrocarbons (PAH) may involve solvent extraction, sample concentration and separation. To assess the rate of vaporisation in a vacuum concentrator with centrifugation function (Concentrator 5301, Eppendorf) changes in the volume of cyclohexane with time were analysed, dependent on the sample volume. Different sample volumes and evaporation times were used: cyclohexane volumes 15, 13, 11 and 9 ml; with times of 20, 22, 24 and 26 minutes. For a second set of samples the volumes were 7, 5, 3 and 2 ml, with evaporation times of 18, 14, 10 and 8 minutes. The pressure in the concentrator was measured periodically over the first 2.5 min.

With the initial volume constant, the evaporation rate, in terms of $\Delta V/t$, decreased with evaporation time increased and also decreased on increasing the number of samples in a set. With the time kept constant, the rate fell with decreasing sample volume. This was ascribed to the effect of changes in the pressure inside the vacuum concentrator. The results and their interpretation will serve as a basis for optimising the evaporation so as to obtain the required sample volume in relation to the initial volume. In order to assess the change in the concentration of PAH in typical analytical samples during solvent evaporation, known amounts of PAH were added to cyclohexane samples. After concentration these samples were analysed using the GC-MS method, but to the vials from which all solvent had evaporated, 1.5 ml of cyclohexane were added before analysis. It was shown that during solvent evaporation there was no significant loss of PAH.

CHROMATOGRAPHIC CHARACTERIZATION OF METALLO-DERIVATIVES OF PROLINE-TOLYLPORPHYRIN

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The synthetic porphyrins, especially their metallo-derivatives are among compounds which are subjects of investigations of various processes eg.: photosynthesis, breathing process or photodynamic therapy [1,2].

The purpose of my research was chromatographic and spectroscopic investigation of formed metallo-derivatives of proline-tolylporphyrin. Densitometric method can be successfully applied as a very useful tool (along with the more sophisticated HPLC method) for quantitative and qualitative determination of the chemical reaction products.

In the present work, initial compounds were synthesized by coupling of protected (from the N and C end, respectively) proline with proper tolylporphyrin in the presence of DCC [3]. The next step was the insertion of metal ions into the free base porphyrins, according to the well-known procedure, using as a solvent DMF [2]. The following, synthesized derivatives were investigated:

(1) TTPCONH-Pro-bzl (Fe III)
(2) TTPCONH-Pro-bzl (Co II)
(3) TTPNHCO-Pro-Cbz (Fe III)
(4) TTPNHCO-Pro-Cbz (Co II)

Purification were conducted on chromatography column (30x2 cm), using as an eluent chloroform. The reaction mixture were spotted on silica gel plates as chloroform solution and developed on 10 cm way. The developed plate was placed into densitometry chamber and scanned with 420nm light. Structure of investigated compounds was proved by UV, IR, NMR, MS spectroscopy.

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APPLICATION OF MODIFIED DOUBLE INTERNAL STANDARD METHOD IN DISTILLATION AND EVAPORATION AS SAMPLE PREPARATION IN GC ANALYSIS

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Various procedures of sample preparation prior to quantitative chromatographic analysis always are accompanied with more or less losses of target analytes. Incorrect sample preparation can be resulted in errors which cannot be eliminated by application of the most advanced analysis techniques and data processing.

The modification of the double internal standard (DIS) method was offered earlier [1]. It implies the using as standards not any related substances, but the previous (x-1) and the subsequent (x+1) homologues of analyzed substance (x). This modification permits us to obtain precise results in spite of significant losses of analytes like it is in liquid-liquid extraction, head space analysis, distillation of volatile substances, and evaporation of volatile solvents.

The series of model experiments with evaporation (open phase transition process; concentration in the rests) and distillation (concentration in the condensates) were carried out to compare the results.

Model experiment. The solution of *m*-xylene in 50 ml tetradecane (C=8.6 mg/ml) has been concentrated before GC analysis by distillation (10 ml heptane as low-boiling solvent were added; collected amount of condensate was about 5-6 ml). Standards (multi-row homologues): toluene (C=8.7 mg/ml) and 1,3,5-trimetylbenzene (C=8.6 mg/ml). GC analysis was carried out with packed column 2m x 3 mm with 10 % Carbowax 20M on Chromaton N-AW, temperature programming from 70 ^{0}C up to 140 ^{0}C, ramp 7 ^{0}C/min.

Result:

Component	Average peak areas, S, mV×ms×10^{-3}
Toluene (standard, x-1)	1.13 ± 0.10
m-Xylene (target analyte, x)	0.54 ± 0.04
1,3,5-Trimetylbenzene (standard,x+1)	0.26 ± 0.02

Determined concentration of *m*-xylene 8.7 ± 0.2 mg/ml. Error: +0.1 (s_r = +1.2%)

The application of DIS-method at evaporation of volatile solvents requires further improvement of physicochemical models and algorithms of data processing.

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THE TLC SEPERATION OF SOME ISOMERIC DITHIINODIAZINES AND DIAZINYL SULFIDES

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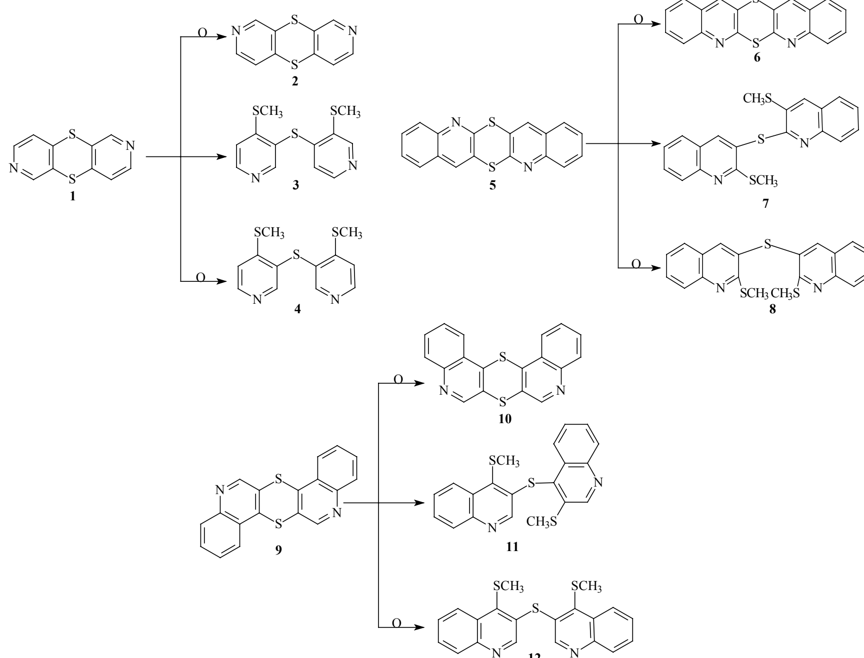
During our study on azinyl sulfides we found unprecedented S→S type of the Smiles rearrangement observed during the 1,4-dithiin ring opening in dithiinodiazines with sodium alkanethiolates. The rearrangement enables isomerizations of dithiins 1, 5 and 9 to dithiins 2, 6 and 10. These opening reactions led also to isomeric diazinyl sulfides 3/4, 7/8, and 11/12, depending on the reaction conditions [1-4]. It was very important to be able to observe the progress of the reactions by the TLC analysis. Therefore, we looked for chromatographic conditions which afforded possibilities for separation of the isomeric compounds.



We revealed a regularity in retention factors of dithiinodiazines. Dithiins of the C_{2h} symmetry (1, 5 and 9) showed higher R_f values than those of C_{2v} symmetry (2, 6 and 10).

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APPLICATION HPLC AND DENSITOMETRY METHOD FOR INVESTIGATION OF REACTION FORMATION OF PORPHYRINS

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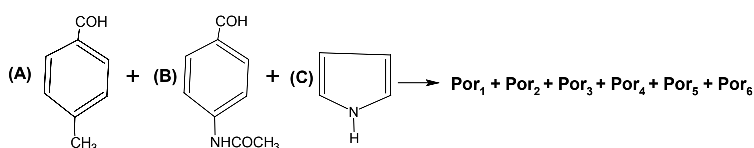
Our earlier work were devoted to investigating of *meso*-tetraphenyloporphyrin derivatives. Acetamidophenylporphyrins were synthesized by the conventional method of *Adler* [1] using 4-methylbenzaldehyde and 4-acetamidobenzaldehyde with pyrrole in the boiling propionic acid:



Three reactions with different ratios of the aromatic aldehydes and pyrole were conducted[2,3]:

3**A** + 1**B** + 4**C**,

2**A** + 2**B** + 4**C**,

1**A** + 3**B** + 4**C**.

The studied substances were separated on silica gel column (2 x 40 cm) with chloroform-methanol v/v 9:1 as eluent. TLC was performed on silica gel type Kieselgel 60 F254, 0.2 mm layer thickness. Mobile phase was the mixture chloroform : methanol v/v 9.5:0.5. The spots have been treated by the densitometric method. Densitograms were obtained by means of the Desaga CD 60 densitometer controlled by Pentium computer. The plates were scanned at λ = 420 nm. In order to confirm the qualitative TLC and densitometry results of HPLC method was used as competitive technique.

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REVERSED-PHASE LIQUID CHROMATOGRAPHY FOR FREE AND PROTEIN AMINO ACIDS FROM SPECIMENS OF ANIMAL ORIGIN*

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Key words: amino acids, seleno-amino acids, reversed-phase HPLC

Introduction: The ability to separate and quantitate amino acids (AAs) has numerous applications in clinical and protein chemistry. These procedures resolve most AAs with satisfactory detection limits, particularly if *o*-phthaldialdehyde (OPA) is applied as the *post*-column derivatizing reagent. Unfortunately, *post*-column techniques mostly require specialized and expensive equipment dedicated only to AA analysis. Therefore, it seems to be useful to develop the simple high-resolution reversed-phase liquid chromatographic method (C_{18}-HPLC) procedure with *pre*-column derivatization for the analysis of protein- and free-AAs in physiological fluids as a cheaper alternative to commercial amino acid analyzers.

Experimental: For the separation of derivatized amino acids (AAs) an 2690 Alliance separation module with a Waters 996 photodiode array detector (DAD) and a Waters 474 fluorescence detector were applied. OPA-AA derivatives were detected using DAD operated in a UV range from 195 to 400 nm. Fluorescence detections were taken at the optimum excitation and emission wavelengths at $\lambda_{ex}/\lambda_{em}$ = 231/470 nm (FD-1) and $\lambda_{ex}/\lambda_{em}$ = 336/425 nm (FD-2). An analytical column used was the Nova-Pac C_{18} column (4 µm, 250x4.6 mm, Waters). For analysis of derivatized AAs in all assayed biological samples, the quaternary gradient elution program was used at the column temperature of 37°C.

Results: The new HPLC procedure resulted in system pressure changes (from 25 to 36 MPa) in accordance with chromatographic rules, excellent OPA-AAs peak shapes, close to symmetrical even with elution times to 61 min and such background fluctuations witch permitted accurate and precise quantitate all OPA-AAs. Twenty four amino acids peaks as OPA derivatives were differentiated from unidentified species in plasma blood or liver and femoral muscles of rat by the use DAD detector (at 231 and 336 nm), as well as fluorescence monitoring (FD-1 and FD-2). No co-elution of OPA-AAs with unindentified endogenous species in all examined specimen of biological origin was observed for DAD monitoring in the spectral range of 205–370 nm and also FD-1 and FD-2. The satisfactory accuracy of all OPA-AAs derivatives and purity was confirmed by comparison UV-DAD spectra of OPA-AAs of all assayed biological samples with UV-DAD spectra of OPA-AAs in processed standards. Considering the results of UV-spectra comparison it is reasonable to conclude that all OPA-AAs peaks were pure in the UV range of 212–370 nm and avoiding co-elution with endogenous species in examined samples. All OPA-AAs peaks were absent from the blank, when the current HPLC procedure was applied.

Conclusion: The chromatographic procedures for the assays of free and protein primary AAs described show the advantages of the *pre*-column derivatization method. Elimination of the *post*-column reactors yield a cheaper and less time consuming, and a more versatile analytical tool. Accurate and rapid analysis of AAs and especially more stable of OPA-AA derivatives was achieved. Our new chromatographic method is most suitable for simultaneous determination of Se-Cys, Se-Met, h-Cys or/and DAPA (traces of bacteria protein) together with other free or protein AAs. The minimum detectability for AAs could be improved by exciting the OPA-AAs derivatives at 336 nm or the UV detection at 231 nm and by applying more concentrate processed samples (two-threefold). A long and high resolution C_{18}-column enabled satisfactory routine separation and quantify of all OPA-AAs from endogenous species present in liver, muscles and blood serum samples.

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RETENTION BEHAVIOUR OF QUINOLINES ON C 18 AND CN-PROPYL STATIONARY PHASES BY HPTLC

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Heterocyclic bases indicate wide spectrum of biological activity because of their molecular structure and possibilities of various interactions with receptors. Therefore they are subject of scientific interest and often subject of analyse. Heterocyclic bases appear in solutions as ionized and unionized forms and therefore they can interact in chromatographic systems as ions and as neutral molecules. It causes strong diffusion of bands in RP systems and therefore tailing, unsymmetrical spots and poor efficiency. Especially the presence of free surface silanols on the silica based stationary phases gives the risk of ion-exchange interactions with basic molecules.

Silanol interactions can be reduced by: use a low pH mobile phase to suppress silanol ionisation; use a high pH mobile phase to suppress investigated basic compounds ionisation; addition of ion-pairing reagents to purpose create neutral associates; addition of a silanol blockers to the eluent, or selection a stationary phases.

Some standards of quinoline were chromatographed on RP 18 and CN-silica adsorbents using various aqueous eluents: buffered aqueous eluents at different pH, eluents containing anionic ion-pairing reagents or different amines as silanol blockers. The effect of kind and concentration of organic modifier, ion-pairing reagents or silanol blockers on retention, separation selectivity, spots shapes and system efficiency were examined.

In eluent systems containing the mixture of organic modifier-water asymmetric spots and very low efficiency were obtained. To improve spots' shape and system efficiency buffered mobile phases were applied, but spots were still asymmetric and tailing. The most symmetric spots were obtained in eluent system containing phosphate buffer at pH 3 or 4, when the dissociation of free silanols was suppress. Afterwards the effect of addition of ion-pairing reagents (octane-1-sulfonic acid sodium salt, pentane-1-sulfonic acid sodium salt) to elunts was examined. In these systems was observe considerably improvement of spot shapes and increase of theoretical plate number for most causes.

The most symmetric spots and the highest theoretical plate number were obtained in systems containing addition of amines (diethylamine, diethanolamine, triisopenthylamine, trioktylamine, triethanolamine, or tetrabuthylammonium bromide) as silanol blockers in mobile phase.

APPLICATION OF SEC AND RP-HPLC TO THE INVESTIGATION MECHANISM OF HYDROGERMYLATION REACTION PRODUCING TRIBENZYLGERMANYL DERIVATIVES

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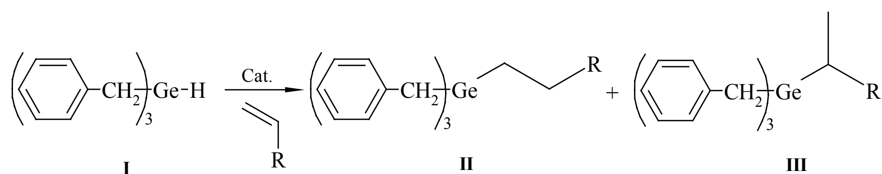
The hydrogermylation reaction is the one of the most convenient way to the direct process in producing compounds with germanium carbon bonds. Tribenzylgermane compounds of general formula II or III have been synthesized.



R = $-CH_2C{\equiv}N$; $-CH(OEt)_2$

It turned out that the reaction directions and yields depend on its conditions (temperature, time, concentrations *etc.*) and used catalyst ($H_2PtCl_6{\cdot}6H_2O$ or PtO_2). Size exclusion chromatography and reversed phase HPLC with diode array spectrophotometric and spectrofluorometric detectors have been used to study these mechanisms. It was found good correlation between chromatographic peak areas recorded at 265 nm (wavelength characteristic for C≡N group) and predicted reaction pathways, confirmed by NMR spectra. Additionally, side reaction have been studied at 240 nm (characteristic wavelength for tribenzylgermane group). From the other side good, selective chromatographic separations were obtained using fluorescence detector at 232/240 nm.

DETERMINATION OF ORGANIC LEACHABLES FROM COMMERCIAL RESIN-MODIFIED GLASS-IONOMERS BY MEANS OF HPLC-MS

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Elution of organic compounds from resin-based dental fillings during their application in the human mouth environment may have a potential impact on the human health. Ethanol, water and other solvent very often present in human mouth have ability for penetration of dental filling placed in the human tooth. Penetration of liquids into the tooth may lead to the liberation of unreacted dental filling ingredients or their degradation products. Determination of these compounds is necessary for better knowledge from possible harmful effects caused by dental fillings. The aim of this study was the isolation and identification of compounds released from resin-modified glass-ionomer cements RMGICs (resin-based dental materials applied in dentistry). Compounds were extracted from fillings by using three solvents (40% ethanol, water, acetic acid and artificial saliva). Liquid samples containing leached compounds were then extracted, evaporated and analyzed by using of HPLC-MS (high-performance liquid chromatography-mass spectrometry) and HPLC-DAD (high-performance liquid chromatography-diode array detection) techniques. Almost twenty components (monomers and additives) of RMGICs were identified. The main identified leachables were: Bis-EMA (ethoxylated bisphenol A dimethacrylate), UDMA (urethane dimethacrylate), TEGDMA (triethylene glycol dimethacrylate), HEMA (2-hydroxyethyl methacrylate) as monomers and camphorquinone (photo-initiator), tert-butyl-p-hydroxyanisole (inhibitor), 4-(dimethylamino) ethyl benzoate (co-initiator) as additives.

This work was supported by Ministry of Education and Science grant Project No 1 T09B 089 30 what is gratefully acknowledged.

RETATION BEHAVIOUR OF FLAVONOIDS DERIVATIVES ON CYANO SILICA GEL LAYERS

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Flavonoids are a natural water-soluble plant pigments. They all show a similar chemical construction including two benzene rings that lie on either side of 3-carbon ring.

The various classes of flavonoids are created by multiple combinations of hydroxyl groups, sugars, oxygens and methyl groups.

According one system flavonoids are divided into the groups:flavonols, flavones and flavonones.

Flavonoids occuring in medical plants, compounds which are frequently analysed owing to their anti-allergenic, anti-inflammantory, anti-cancer, vasoprotective and radical-scavenging properties. In addition flavonoids act as powerful antioxydants, provading remarkable protection against oxidative and free radical damage.

In this work TLC method was used to analysis 20 flavonoid standards. To characterize reparation of flavonoids, relationships between Rf and modifier concentration were determinated for cyanopropyl silica. The mobile phase was dichloromethane, acetonitrile, formic acid (6:4:0,05 v/v)- mixture A with water. Content the water of the mobile phases was 7,5% (v/v).

Plates were developed in horizontal DS-Chambers for TLC (Chromdes, Lublin, Poland). They spots were detected by UV illumination at 254nm or 366nm.

DETERMINATION OF BIOACTIV REDUCTORS BY MEANS OF OXIDATION OF LEUCOCRYSTAL VIOLET

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A simply and accurate analytical procedure for determination of bioactive reductors after chromatographic separation was proposed. Optimum conditions for TLC separation of ana-lytes were established. The UV (254 nm) detection of bioactive reductors was used. Spectro-photometric quantification was by means of a sensitive and simply method based on oxidation of leucocrystal violet to the crystal violet. The absorbance of the crystal violet dye formed was measured at 588 nm. The procedure was successfully applied to the determination of analytes in pharmaceuticals. The results were in good agreement with the declared amounts as well as with those obtained by reference method.

The suggested reactions are:

1. The oxidation of the analyte by the added iodate (V) anion.
2. The iodide ions reacted with the excess of iodate (V) ions in acidic medium (addition of HCl) to form free iodine.
   $5I^- + IO_3^- + 6H^+ \rightarrow 3I_2 + 3H_2O$
3. The iodine oxidized leucocrystal violet (LCV tris(p-dimethylnitrophenyl)-metane) to the coloured crystal violet (CV^+).

TLC DETERMINATION OF DECOMPOSITION PRODUCTS OF TETRAPHENYLPORPHYRIN DIAZONIUM SALT

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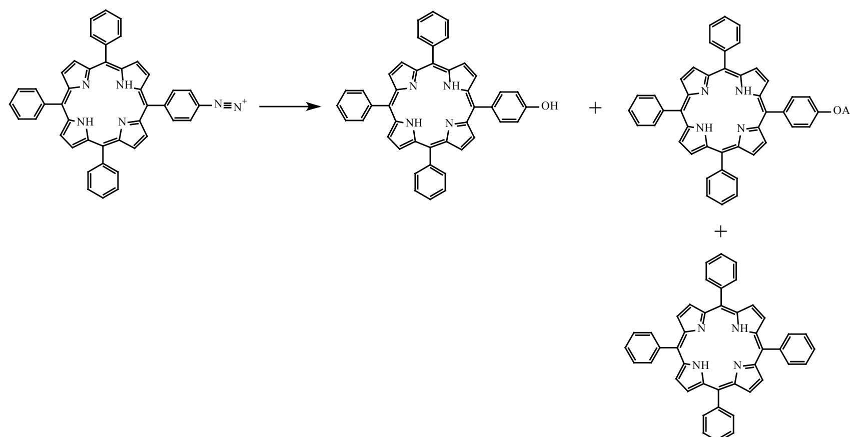
Controlled modification of porphyrin phenyl substituents usually results in changes in physicochemical and chromatographic properties of all mother compounds.

During last year we have been interested in the synthesis of mono-para-hydroxy-phenyl derivative of TPP using the mono-para-aminophenyl derivative of TPP as a starting material. Nitro derivative we obtained by direct nitration of TPP, using sodium nitrite in TFA solution. Purification of the product was conducted by column chromatography. Stannous chloride in concentrated hydrochloric acid was used for the hydrogenation of nitro group into an amino unit. The amino group was converted to diazonium salt. The diazonium group was replaced by the OH group. During this reaction two another products were obtained. These products were detected and characterized by TLC and spectrometric methods.

Here we summarized results of our studies on isolation and purification of all products of diazonium salt replacement in tetraphenylporphyrin derivatives.



SEPARATION AND DETECTION OF CONJUGATED LINOLEIC ACID ISOMERS AND FATTY ACIDS FROM RUMINAL FLUID INCUBATED *IN VITRO**

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Introduction: In recent years, there has been an increased interest in ways to manipulate the fatty acid (FA) composition of meat. EPA, DHA and the ratio of n-6:n-3 PUFA is particularly beneficial in ruminant-derived meat, milk and dairy products obtained especially from animals that have consumed grass which contains high levels of C18:3. Ruminants also naturally produce conjugated linoleic acids (CLA) isomers which may have a range of nutritional benefits in the diet. Unfortunately, the ruminal digestion of lipids results in major changes of the profile of FAs due to biohydrogenation of unsaturated FAs. Therefore, the influence of dietary additives, such as vegetable or fish oils, free FAs and essential minerals, on biohydrogenation of dietary unsaturated FAs has been studied *in vivo* and *in vitro*. Here we describe improved procedures for the analysis of the complex FA mixture in ruminal digesta.

Experimental: Eight ruminally fistulated adult sheep received a mixed diet comprising grass hay, barley, molasses, soybean meal, minerals and vitamins, fed in equal meals of 500 g at 8 and 16 h. Ruminal digesta samples were taken before feeding in the morning from each sheep and then strained through linen cloth for analysis. One ml of ruminal fluid was added under CO_2 to tubes containing 0.1 ml of 50 g/l fish oil (FO) and 0.1 ml of 20 g/l linoleic acid (LA). The tubes were incubated for 18 and 24 h at 39 °C. Extraction and derivatization of FAs were carried out using procedures based on those described by Christie [1]. Samples were mixed with 1.25 ml of acidified salt solution (17 mM NaCl in 1 mM H_2SO_4) and 2.5 ml of methanol. The mixture was vortexed for 1 min, then 2.5 ml of chloroform with added 0.2 mg/ml BHT were added and the mixture was vortexed for 2 min. The lower layer was dried and then solvent was evaporated. The dried extract was re-suspended in 0.5 ml of toluene, the suspension was vortexed, then 1 ml of 1% H_2SO_4/methanol was added. The tube was flushed with N_2 then closed and incubated at 50°C for 1 h. The tube was cooled, 2.5 ml of 5% NaCl were added, the tube was vortexed, then 1 ml of hexane was added and the tube was vortexed again. When layers had formed, the upper layer was transferred to a fresh tube and the hexane extraction was repeated twice on the lower phase. The hexane fractions were pooled and 1.5 ml of 2% $KHCO_3$ was added. The upper layer was removed, evaporated, re-suspended in 0.2ml of hexane/BHT, then transferred to a GC vial. The gas chromatograph was an Agilent 6890 instrument (UK) equipped with a Varian CPSil88 column, 100 m × 0.25 mm with a film thickness of 0.2 µm (Varian). The temperature programme was as follows: 80 ^{o}C for 1 min; 25°C/min to 160 ^{o}C then hold for 3 min; 1C^{o}/min to 190 ^{o}C then hold for 5 min; 2C^{o}/min to 230°C then hold for 25 min. The carrier gas was He and the column was operated at constant pressure (20 psi) with a flow rate of 0.5 ml/min. The injector held at a temperature of 275°C.

Results: The main analytical problem in the present study was to obtain suitable fractionation of mono-enoic acids (mainly C18:1), FAs containing conjugated double bonds (CFA) and long-chains PUFA (i.e pos-sessing from 20 to 24 carbon atoms). Satisfactory fractionation and values of peak area to noise for all most important FAs as wells as *cis9trans11*CLA, *trans10cis12*CLA and other *cis,cis*CLA, *trans,trans*CLA isomers were obtained using proposed the temperature programme, FID and MS detection and 1:15-1:30 split mode. Combination of gentle derivatization and extraction procedure and long-capillary GC column enabled quantification of precursors of CLA isomers (mainly *trans11*C18:1) and bacterial metabolites of linolenic acid containing conjugated double bond (*cis9trans11cis15*C18:3, *trans9trans11cis15*C18:3, *cis7trans9cis13* C16:3 and *trans7trans9cis13*C16:3) and a non-conjugated metabolite of linolenic acid (i.e. *trans11cis15* C18:2). The chemical structure of all detected FAs, CLA isomers and CFA was confirmed using MS detection and silver-ion liquid chromatography with UV detection.

Conclusion: The current derivatization and extraction procedures with long-capillary column gas-chroma-tography provide accurate, sensitive and high-resolution analytical tools for routine simultaneous quantifi-cation of complex FAs mixtures from specimens of ruminal digesta origin.

Supported by the Marie Curie Training Sites 'Mass School'

Reference:

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MODIFIED ANALYTICAL METHOD FOR POLYCYCLIC AROMATIC HYDROCARBONS USING RP-HPLC WITH FLUORESCENCE DETECTION AND GPC FOR SAMPLE PREPARATION AND ITS APPLICATION FOR DIFFERENT FOOD SAMPLES

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A HPLC, with the fluorescence detection, method for the determination of eight polycyclic aromatic hydrocarbons (PAH) with four to six condensed aromatic carbon rings in edible oils and smoked products has been developed. The method implies preparative size exclusion chromatography for efficient one-step lipid removal without saponification and benzo[b]chrysene, used as an internal standard, for quantification. Two other methods (liquid-liquid extraction and solid phase extraction) for one-step clean up and sample enrichment were tested. However, it turned out that one-step procedures did not remove lipids completely. The calibration curves showed a good linearity for all PAH in the concentration range 0.1÷100 ppb. The repeatability (RSD, n =6) of different PAH ranged from 0.5 to 5%. The analysis of standard reference material of the National Institute of Standards & Technology (mussel tissue, SRM 2978), Community Bureau of Reference (coconut oil, CRM 458) and Central Science Laboratory (olive oils, FAPAS 0615, 0618 and 0621) resulted in a good accordance between measured and certified concentrations. The elaborated method has been applied to the determination of PAH contents in 12 samples of edible oils, rape seed, milk powder, hens white, egg yolk and smoked sausage, white cottage cheese and sprats. The proposed method is selective and sensitive enough for the determination of PAH in different food matrix. It made this assay suitable for routine analysis. The study highlights the use of fluorescence excitation and emission spectra to evaluate peak purity PAH identifications were obtained using fluorescence spectra. It turned out that the concentration of PAH in edible oils obtained by cold pressure (so called ecological food), without further purification, exceed maximum value proposed by EU directive.

AN APPROACH FOR THE DETERMINATION OF CHLORO-CATECHOLS, CHLOROGUAIACOLS AND CHLOROVERATROLES IN BLOOD SAMPLES

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Determination of chlorinated aromatic compounds persistent in sediment, soil and water matrix has been widely discussed. Some of these compounds such as: chlorophenols, chlorocatechols, chloroguaiacols and chloroveratroles are known as components of pulp bleaching effluents. Their persistence in aquatic environment proved to be dangerous for many species of fish and plants, because of their endocrine disrupting properties. Since chlorine is substituted by oxygen or hydrogen dioxide during bleaching pulp and paper, chlorinated aromatic compounds are no longer formed. Many of them are still present in the environment as a degradation products and may be transformed into dioxins during spontaneous processes, as for example combustion. This occurrence illustrates the importance of developing of the methods for determination of chlorinated organics as for example: chloroveratroles, chlorocatechols and chloroguaiacols in many matrices.

Although, the increasing interest of the determination of those compounds in different matrices caused a new analytical procedures and techniques creation. The aim of this work was to develop the derivatization and chromatographic analysis for the simultaneous determination of chloroveratroles, and pentafluorobenzyl bromide (PFBBr) derivatives of chlorocatechols and chloroguaiacols by GC- ECD and GC-MS in whole blood samples.

Derivatization was conducted in the presence of triethylamine as a proton acceptor reagent and the obtained derivatized standards were used for estimation linear range, detection limits and precision. The reaction conditions and chromatographic analysis processes were optimized. Further efforts were put on developing of the analytical procedure for the determination of these compounds in blood samples. Many different solvents and their mixtures such as acetone-hexane, isopropanol-hexane, acetonitrile, methanol, methanol-acetone-hexane, HCl, were applied as a denaturation solvents in recovery experiments. Each sample was then extracted using hexane/methyl-tertbutyl ether mixture. After evaporation the sample was derivatized with PFBBr, and cleaned using acidic and neutral silica-gel columns and Alumina columns. However, the calculated recovery values shown acceptable levels, the method needs an improvement in the matrix separation using selective methods as for example GPC technique.

TLC DETECTION OF SELECTED STEROIDS USING PMA STAINING METHODOLOGY

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This work is continuation of our earlier contribution concerning optimization of TLC detection of steroids using phosphomolybdic acid (PMA) staining methodology. In this study detection of ergosterol, stigmasterol, cholesterol, dihydrocholesterol, 4-cholesten-3-one, 7-dehydrocholesterol and cholesterol acetate was investigated. The selection of our component of interest was due to high transparency in UV light and their potential application as the environmental biomarkers for endocrine disrupting phenomenon.

Steroids were separated on glass plates coated with silica gel and mobile phase composed of methanol/dichloromethane 5:95 (v/v). After separation TLC plates were sprayed with 10% (w/v) PMA in methanol and heated at different temperatures (from 40 to 120°C) and times (2-40 minutes). Spots intensities were evaluated using TIFF 8-bits images and Scion Image software. The best conditions for high signal intensity was determined using 3D-maps generated form the raw experimental data points. It has been found that similarly to bile acids [1] a quantitative effect of PMA dyeing is strongly time/temperature dependent and best conditions for robust detection can be expected if the plates are heated in the temperature between 60 and 80C for time more than 10 minutes. Under such condition this technique is capable to determine ng/spot quantity of steroids investigated.

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PHYTOCHEMICAL AND GENETIC ANALYSES OF *Salix purpurea*

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The bark of the willow tree (*Salix*) species has been traditionally applied as painkiller and anti-inflammatory means in Europe. Willows and other *Salicaceae* plants species usually contain secondary metabolites phenolic glycosides in their bark. The concentration of these components may vary among individuals and this variability is expected to be found in genetic profile. RAPD (Random Amplified Polymorphic DNA) is one of the simplest and fastest methods of genetic fingerprinting which is widely used also for plant genome studies as well [1,2].

We have attempted to find some relationship between genetic and phytochemical fingerprinting obtained from RAPD and GC analyses. In this study we applied rapid a method of a row bark extract principles analysis. The willow bark compounds were extracted in cold methanol with sonification and analysed by high-resolution capillary gas chromatography [3,4]. RAPD analyses were performed for a group of plants belonging to the willow genus using eight decamer primers.

This paper describes methods, which have been tested and modified for genetic/phytochemical correlation studies. Genetic distance matrix for RAPD results was obtained according to the Sneath-Sokal / Adenberg algorithm which was chosen after PTP test of binary data, and subsequently tree diagrams were drawn and PCA plot as well.

A statistical analysis of both, phytochemical and genetic data, revealed a possibility preliminary estimation of the phytochemical profile on the base of genetic data.

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A VALIDATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR QUANTITATIVE ANALYSIS OF CARBAMAZEPINE IN HUMAN SERUM

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Carbamazepine is one of the most widely prescribed antiepileptic drugs and is also used in the treatment of trigeminal neuralgia and psychiatric disorders.

The aim of this work was to develop HPLC method for the determination of carbamazepine in human serum. The method is simple with a one-step extraction procedure (liquid-liquid extraction with dichloromethane and 1M NaOH), isocratic HPLC separation, and UV detection at 210nm. Use phenacetin as the internal standard provided good accuracy without interference by endogenous compounds. Chromatographic separations were performed on the analytical column (250 mm x 4 mm) filled with Nucleosil 100 C-18 of 5 μm particle size. The mobile phase was a binary mixture of acetonitrile and water (40:60 v/v). The flow-rate was 1.2 ml/min. Under these conditions the retention times of carbamazepine and phenacetin were: 5.5 min and 4.1 min, respectively. The method has been validated for accuracy, precision, selectivity, linearity, recovery and stability. The quantification limits for carbamazepine in serum was 50 ng/ml, limit of detection was 20 ng/ml. Average extraction efficiencies of carbamazepine was 82 %. Linearity of the method was confirmed for the range of 200-5000 ng/ml ($r = 0.9998$).

Within-day and between-day precisions, expressed as the relative standard deviation (RDS%), ranged from 7.2 to 0.5% and 5.4 to 2.6% for carbamazepine. The described method was successfully applied in pharmacokinetic studies for routine monitoring of active substance after single oral administration of a 200 mg dose of carbamazepine. Studies were performed on 20 healthy volunteers of both sexes.

Proposed HPLC method is sensitive, accurate and rapid and could be a reliable alternative to other separation method for the analysis of those drugs. The pharmacokinetic experiments were performed on an equal male to female group of volunteers therefore the pharmacokinetic parameters ($AUC_{0-\infty}$, AUC_{0-t}, C_{max}, T_{max}, K_{el}, $T_{0.5}$) are probably more realistic.

MELANIN FROM THE HUMAN *Melanoma malignum* AN OPTIMIZATION OF THE PIGMENT ISOLATION PROCEDURE

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Melanoma malignum is one of the most malignant skin tumors and the incidence of this disease tends to rise. Among various forms of human melanoma, most are pigmented with melanin. For this reason, taking up the investigations in search of potential correlation between melanin structure and the tumor type or even progression of neoplastic process seems to be useful.

One of the most significant limitations in structural studies of naturally occurring melanins is the lack of appropriate procedures that allows isolation of intact pigment being free from tissue contaminants, in particular proteins and lipids.

In the presented study, melanin pigment was isolated from the human *melanoma malignum* tissue with the use of three different enzymatic methods, such as modified by the authors methods of Double [1] and Wilczek [2], and the method described by Chomczyński [3]. The isolation procedures used were compared as regards contamination of the isolated pigment with proteins and lipids. The melanin purity was evaluated by thermochemolysis in the presence of tetramethylammonium hydroxide (TMAH). Synthetic eumelanin obtained by tyrosinase-catalyzed polymerization of DOPA was used as the reference material.

The highest purity was achieved for the melanin biopolymer isolated according to the modified method of Double. The products characteristic for thermally degraded eumelanin-type pigments dominated pyrolytic profile of this sample, whereas the levels of protein- and lipid-originated products in the pyrolysate were relatively low.

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THE CAPILLARY ELECTROPHORESIS MICROCHIPE WITH ELECTROCHEMISTRY DETECTOR FOR THE DETERMINATION OF DOPAMINE AND NORADRENALINE

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Microfabricated fluidic devices, integrating the sample-handling processes and measurement step onto microchip platforms, are of considerable recent interest. The advantages of a microchip include lower consumption of solvent and reagent, shorter analysis time, portability and disposability.

In addition to the commonly used optical detectors, electrochemistry (EC) detectors seem to be another excellent option for capillary electrophoresis measurement raised from the advantages of relatively low cost, high sensitivity and excellent suitability for miniaturization.

But the use of the CE-EC detectors has been limited by a number of problems: the interference of a high separation electric field can affect the stability of the EC background current, and the position of working electrodes from the outlet of the CE column can affect the reproducibility of EC measurement.

We have fabricated and evolution of fully integrated EC-detector *(two electrode and decoupling device on the same glass substrate)* in CE-microchip on the glass substrate, which is reversibly sealed with a PDMS replica containing the injection and separation channels.

The thin gold films with Ti adhesion layer served as the working electrodes of the amperometric detection.

In current CE-EC microchips, the reference electrode (Ag/AgCl) and counter electrode are externally inserted into the channel outlet reservoir to offer a stable reference potential. In this study gold electrode serving as the pseudoreference electrode was simultaneously miniaturized and integrated on the CE microchip and close to the working electrode in order to decrease the potential shift during amperometry detection. The stability of this electrode and effects on EC detection was estimated.

The use of decoupler has been shown to decrease the interference of the electrophoretic current. The solid film integrated directly into the CE microchip across the separation channel by microfabrication techniques and located in front of the EC electrodes, was developed. We have estimated the decoupling efficiency of gold and palladium materials and the effect of spacing between decoupler and working electrode on amperometric detection sensitivity.

The analytical performance of on-chip CE/electrochemistry microchip was characterized for the determination of catechols (dopamine and noradrenaline) in 10 mM MES and 10 mM borate buffer (pH 9,18). Variables influencing the separation efficiency and amperometric response, including the channel-electrode spacing, separation voltage and detection potential were optimized.

APPLICATION OF HEADSPACE SPME AND ENANTIOSELECTIVE GC IN CONIFER'S VOLATILE COMPOUNDS STUDIES

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SPME - Solid Phase Micro extraction is a method of volatile compounds extraction [1]. One of its most important advantages is the reduction of analysis duration in comparison to conventional extraction and distillation methods. This method has wide range of applications in analysis of volatile compounds present in food [1], alcohols [2, 3], biological fluids, soil, water and other materials. SPME-GC was also successfully applied in analyses of compounds belonging to terpenoids present in aromatic plants [4]. The main compounds present in needles of *Pinus sylvestris, Picea abies* (*Pinaceae*) and *Juniperus communis* (*Cupressaceae)* are the monoterpenes, which constitute for 60-70% of their essential oils. Most of the monoterpenes are optically active and can occur in nature in two enantiomeric forms – (+) and (-).

Thus the headspace SPME technique coupled with enantioselective GC was applied for analysis of volatile monoterpenes from the mentioned conifer plants. Headspace SPME directly from oil glands (present in needles of all three plants and juniper cone berries) was applied as an alternative to time consuming essential oil water distillation. 100µm PDMS fibber (Supelco) was used to absorb the volatile compounds.

Headspace SPME-GC is a fast and simple method used in enantioselective analyses of a wide range of coniferous plant materials like dried or fresh needles (*Juniperus communis* L., *Picea abies* (L.) H. Karst., *Pinus sylvestris* L.), young shots (*Pinus sylvestris* L.) and cone berries (*Juniperus communis* L.).

The amount of used material was very insignificant (e.g. one cone berry or 100 mg of needles). Therefore it can be applied as a quick diagnostic test in phytochemical evaluation of row material used for pharmaceutical purposes.

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SEPARATION OF SOME TROPANE ALKALOIDS FROM *Datura innoxia* Mill. ON DIFFERENT STATIONARY PHASE BY TLC METHOD

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Alkaloids are widely used in therapy because of their wide biological activity. One of the group of the alkaloids are tropane alkaloids. Some tropane alkaloids known from ancient times were recognized and documented in XVI century. Now more than 200 of tropane alkaloids existing in nature are known. Some of them have synonim names, for example: hyoscine = scopolamine, anisodoamine = 6β-hydroxyhyoscyamine. Tropane alkaloids are the popular substances in nature. There are some plants in all climate zones, containing some tropane alkaloids. Tropane alkaloids can be found in plants from *Solanaceae or Erytroxylaceae* family. *Datura innoxia* Mill. is a common plant material containing tropane alkaloids. Scopolamine – important pharmaceutical product – is isolated from the material industrially in preparative scale. After the isolation the industrial waste material still contains several tropane alkaloids such as: atropine, homatropine, tropine and scopolamine residues, which are valuable as products which can be applied in pharmaceutical industry. Alkaloids as organic bases are difficult task of analysis because their extraction and retention behaviour is often similar.

In the presented paper a possibilities of the separation and identification of the tropane alkaloids from *Datura innoxia* Mill. are demonstrated in TLC chromatographic systems with silica gel, aluminium oxide, and cellulose layers. On SiO_2 layer in pure organic solvents the tested alkaloids stay in the start line, similar as in water-organic mobile phases (in the mobile phases only scopolamine hydrobromide and scopolamine oxide migrated). On SiO_2 layer migration of the tested tropane alkaloids can be obtained in buffer-organic solvent mobile phases. Different pH buffer, and buffers with different ionic strength were tested as mobile phases in SiO_2 layer. Second stationary phase tested for a separation and identification of the alkaloids is Al_2O_3 layer. Only layers of type T are useful for chromatography of the tropane alkaloids. Kind of the stationary phase, organic solvent and composition of the mobile phase were tested in order to obtain a separation of the tropane alkaloids from *Datura innoxia* Mill. The influence of the pH of the water - organic solvent in the pseudo - reversed phases on silica and aluminium oxide layers were also examined.

QUANTITATIVE DENSITOMETRIC DETERMINATION OF AESCIN IN SEEDS AND CAPSULES AT THREE AESCULUS SPECIES

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Aescin is the major active principle from Aesculus hippocastanum (*Hippocastana-ceae*) the horse chestnut tree, a plant widely distributed all over the world. A number of reports dating from the early 18th century have indicated therapeutic properties for horse chestnut. These have ranged from anti-fever to, at the end of the 19th century, anti-haemorrhoidal properties. Presently it has shown satisfactory evidence for a clinically significant activity in chronic venous insufficiency (CVI), haemorrhoids and post-operative oedema.

Mature seeds and capsules of Aesculus hemiacantha and Aesculus marylandica were collected in Botanical Garden of Wraclaw University and the mature seeds and capsules of Aesculus parviflora were collected at Wojsławice Arboretum. All plant material was dried in a dark place, at room temperature.

The HPTLC densitometric detremination was performed directly at 212 nm without using spray or dipping reagents.

Results for the HPTLC densitometric determination of aescin in three Aesculus species.

Sample name	Amount of substance (aescin)	Ratio
Aesculus parviflora seeds	574,26 µg	2,87%
Aesculus parviflora capsules	-	-
Aesculus marylandica seeds	813,77 µg	4,07%
Aesculus marylandica capsules	9,57 mg	47,84%
Aesculus hemiacantha seeds	5,11 mg	25,56%
Aesculus hemiacantha capsules	1,57 mg	7,84%

PHENOLIC ACIDS IN UNDERGROUND PARTS OF *Crithmum maritimum* L.

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The roots of *Crithmum maritimum* L. (*Apiaceae*) were investigated on the presence of phenolic acids. The material was collected in year 2002 in botanical garden in Gdańsk from the cultivated plants (control) and plants fertilized with Tytanit formula. The methanolic extracts (exhaustive extraction in Soxhlet apparatus) of the plants were analyzed. Free phenolic acids and fractions obtained from the acid and alkaline hydrolyses were analyzed by 2D TLC on cellulose layer [1,2]. Next RP-HPLC analysis was performed using mobile phase methanol : water (20:80) with 1% acetic acid (v/v) [2]. Before HPLC samples were purified by SPE. In investigated extracts estimation of content of phenolic acids was done using the Arnova [3] and SPE/HPLC methods. In described procedure the protocatechuic, p-hydroxybenzoic, chlorogenic, vanillic, caffeic, syringic, p-coumaric and ferulic acids were detected and identified in investi-gated extracts. The amount of phenolic acids in plant control was lower then in fertilized plants (10,9 mg/g of dry mass and 13,2 mg/g dry mass respectively). Differences of the phenolic acids content in investigated extracts from plant control in comparison with extracts from plant fertilized with Tytanit formula were observed.

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CHROMATOGRAPHIC METHODS IN THE MEDICINAL PRODUCTS' QUALITY ASSESSMENT

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Before being placed on a market each medicinal product is evaluated during the process of registration. Quality of the product is assessed on the basis of the submitted chemical, pharmaceutical and biological documentation.

Chromatographic methods are applied for the control of active substance's quality. Active substance must comply either with pharmacopoeial monographs or with active substance manufacturer's specification (when no suitable monograph is applicable). Most common chromatographic techniques are HPLC methods applied for related substances tests, assays, in some cases for identity tests and GC applied mainly for residual solvents tests. Thin Layer Chromatography methods (TLC) specified in Ph. Eur. 5.0 monographs are applied mainly for identity tests and several impurities tests. TLC methods are considered insufficient for control of active substance according to ICH and Ph. Eur. requirements and will be successively replaced with new methods, mainly HPLC (Technical Guide for Elaboration of Monographs EDQM) which are capable of identification and quantification of several impurities. In case of "older", not updated monographs, additional validated methods (usually HPLC) must be provided by the active substance's manufacturer to facilitate control according to ICH requirements. In respect to medicinal product chromatographic techniques are applied during product development studies for quality control and stability studies of the finished product. After relevant adjustments methods developed for active substance are applied for quality control of the finished product. HPLC technique is the most commonly used for related substances tests, assays, sometimes for identity tests and for other tests related to pharmaceutical form including dissolution test. Gas Chromatography (GC) is applied mainly for residual solvents tests (solvents used during finished product manufacturing process).

Chromatographic methods are commonly used in assessment of active substances and certain excipients forming pharmaceutical presentations of medicinal products.

THE ELECTROPHORETICAL PROFILES OF BIOLOGICALLY ACTIVE COMPOUNDS FROM DIFFERENT MATRIXES

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The methods of many-component analysis of organic compounds (biogenic amines, steroids, catechines) allows the getting of low limits of detection, high effectiveness and selectivity of analytes separation under the different modes of capillary electrophoresis (CZE, MEKC) were elaborated.

The proposed methods can be used for the development of characteristic profiles (chemical appearance) of biological liquids (urine, plasma and blood serum, brain structures), food substances/drinks (tea, coffee, wine). The comparison of chemical appearance with the standard chemical appearances allows the diagnosis of different diseases and to organize new directions for quality control of food substances.

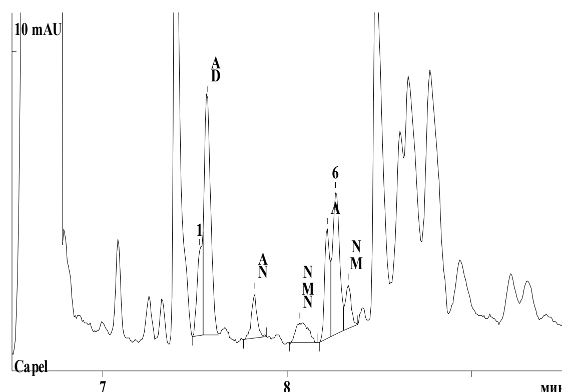

Fig. 1. Electropherogram (specific profile) of urine (healthy volunteer) after SPE on C18

Equipment: Capel 105 (Lumex Ltd. Russia), L_{tot} =60 см, L_{eff} =50 см, $d_{I.D.}$=75 µm. λ=210 nm.
BGE: 1% CH_3COOH, pH 3.0, 30 mM triethanolamine.
Dopamine (DA), noradrenaline (NA), normetanephrine (NMN), adrenaline (A), metanephrine (MN).

The concentration and ratio of biogenic amines changes if the sample is biological liquid from volunteer having different deseases.

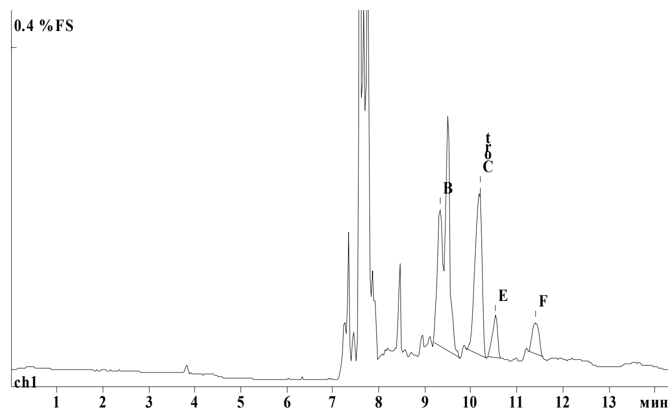

Fig 2. Electrophoregram (steroid profile) of urine (volunteer, “Cortineff” therapy) after LLE with chloroform

Equipment: as Fig 1, λ=254 nm. BGE: 25 mM phosphoric acid, 20 mM SDS, 4,5 M urea, pH 2.5
Corticosterone (B), “cortineff” (Cort), cortisone (E), cortisol (F).

The additional signal of drug was registred and concentrations of basic steroids were increased in comparison with healthy volunteer.

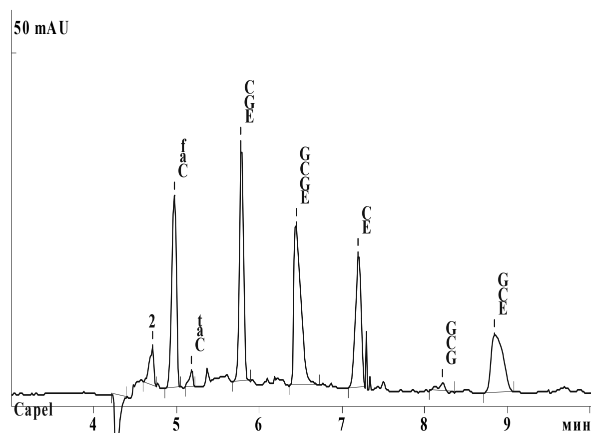

Fig. 3. Electropherogram (specific profile) of green tea extract

Equipment: as Fig 1, λ = 200 nm. BGE: phosphate buffer pH 7.0, 25 mM SDS
Catechin (Cat), epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), gallocatechin gallate (GCG), epigallocatechin gallate (EGCG), caffeine (Caf).
The concentration of basic components is more then the case of black tea.

DETERMINATION OF SERUM PROTEINS BY CAPILLARY ELECTROPHORESIS

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Human blood is extremely heterogeneous with respect to its protein content (325 different proteins), which in most cases are below the detection limit of the standard detectors. A rough analysis of serum proteins is very useful for the diagnosis of several dysproteinemias, such as acute and chronic inflammation, nephrosis, cirrhosis, poly- and monoclonal gammopaties. Protein concentration in blood are tightly controlled to balance their physiological functions in areas such as immunity, coagulation, small molecule transport, and lipid metabolism. Because there are many instances in peptide and protein analysis in which the analyst is limited by sample size, a highly sensitive and selective analytical technique is essentials. Capillary electrophoresis (CE) offers the potential for automating serum protein analysis traditionally performed on standard agarose gel electrophoresis (AGE) and electrophoresis on cellulose acetate membranes (CAE). Presented CE method requires no sample treatment, because the blood serum was only diluted in deionized water. Our experiments were carried out on a Beckman P/ACE system instrument, equipped with a selectable fixed-wavelength UV detector. The temperature was controlled using a fluorocarbon-based cooling fluid (25.0 ±0.1°C), and the voltage was maintained at 8 kV. The resultant electropherograms were monitored at 214 nm. The best results were obtained using a background electrolyte composed with the sodium dihydrogenphosphate (75 mM), adjusted to pH = 1.9 by using ortophosphoric acid. Using a fused-silica capillary 47 cm length x 75 μm I.D., and low-pH buffer solution, CE efficiently separates serum proteins according to their charge-to-mass ratio into few major regions (pre-albumin, albumin, alpha-1(α_1), alpha-2 (α_2), beta (β_1, β_2), gamma (γ)-globulin. The optimized separation conditions allowed the migration of serum protein fractions within 12 min. Capillary electrophoregrams provide comparable or better details than densitometric scans of AGE, since the CE profile represents direct on-line UV detection of protein via the peptide bonds. An alternative electrophoresis technique would be helpful for the analysis of these complex serum profiles, especially in clinical laboratories performing a large number of serum analysis.

GAS CHROMATOGRAPHIC METHOD FOR DETERMINATION OF ISOSORBIDE MONONITRATE IN HUMAN PLASMA

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Isosorbide mononitrate is substance with vasodilating effects, known and successfully used in treatment of coronary disorders and arterial hypertension.

A rapid and sensitive gas chromatographic method with FID detection has been developed for the determination of isosorbide mononitrate in human plasma. Plasma samples (1 ml) were spiked with an internal standard (methyl 4-hydroxybenzoate) and extracted with dichloromethane. The dichloromethane was evaporated to dryness in water bath at temperature 45°C and residue was dissolved in 20 μl of methanol. Portion of 2 μl sample was injected into the gas chromatograph. The GC apparatus (VEGA GC 6000) was operated on splitless injection mode using helium as the carrier gas. The chromatographic separation was carried out on a non-polar cross linked 5% phenyl–95% methyl silicone column (HP-5, length of 30 m, 0.32 mm in diameter). Temperatures of chromatograph elements were set as follow; column 140°C, injection block 200°C, detector 260°C. Under these chromatographic conditions the retention time of isosorbide mononitrate was 4.7 min and internal standard 5.3 min. The total analysis time including column regeneration was 7 min. Analytical data were collected by computer system for data acquisition (Chromatography for Windows CSW).

The method has been validated for accuracy, precision, selectivity, linearity, recovery and stability. The quantification limit for isosorbide mononitrate was 10 ng/ml, limit of detection was 5 ng/ml. Linearity of the method was confirmed for the range of 10-2000 ng/ml ($r = 0.999$). Within-day relative standard deviations (RSDs) ranged from 2.77 to 10.00 % and between day RSDs from 0.61 to 13.86 %.

The reported GC-FID method could be a reliable alternative to other separation methods for the analysis of isosorbide mononitrate. The assay is specific and reproducible. Mean recovery for isosorbide mononitrate was 88 % for human plasma over the range used. The suitability of the assay for pharmacokinetic studies was confirmed by measuring isosorbide mononitrate concentrations in human plasma after administration of a single oral 20 mg dose of isosorbide mononitrate.

STRUCTURAL INVESTIGATIONS OF NEUROMELANINS SYNTHESIZED IN THE PRESENCE OF LIPID COMPOUNDS. A THERMOCHEMOLYSIS STUDY

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Neuromelanin is a polymer pigment that deposits with age in dopaminergic neurons of the human substantia nigra and may be involved in neurodegenerative processes associated with brain aging and Parkinson's disease. The pigment macromolecule consists of the melanin moiety derived oxidatively from the neurotransmitter dopamine and some non-melanin components, such as proteins and lipids. As it has been demonstrated by our recent pyrolysis-gas chromatography/mass spectrometry studies [1], the latter compounds are not only adsorbed, but also chemically bound to the melanin moiety, most probable at early stages of the pigment formation.

In the presented study, the structures of synthetic neuromelanins obtained by 48 h incubation of dopamine in the presence of palmitic acid or bovine brain lecithin in Tris-HCl buffer (pH 7.4) at 37°C were investigated. The melanin precipitates formed were collected by centrifugation and extensively washed with organic solvents to eliminate non-specifically bound lipids. Dry pigment samples were then pyrolysed in the presence of tetramethylammonium hydroxide and the thermochemolysis products were analyzed by GC/MS.

Substantial amounts of fatty acid methyl esters were identified in the analyzed pyrolysates. The results obtained indicate that lipid compounds present in the synthesis medium during non-enzymatic oxidation of dopamine are chemically incorporated into the growing melanin polymer.

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THE METHOD OF IDENTIFICATION OF LABILE MONOSACCHARIDE COMPONENTS OF BACTERIAL ENDOTOXINS

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Lipopolysaccharide (LPS), also called endotoxin, is a heteropolymer composed of not only neutral monosaccharides but also uronic acids and amino sugars. These labile structures are decomposed during derivatization to alditol acetates or partially methylated alditol acetates, therefore their identification by GLC-MS technique is hindered. During the hydrolysis of glycosidic linkages/bonds an irreversible lactonization of uronic acids or their conversion to aldobiuronic acids may occur. These undesired effects may be minimized by the use methanolysis instead of hydrolysis. Methyl glycoside products are next acetylated. Unfortunately, this procedure has some drawbacks, because several peaks for a monosaccharide are observed on the chromatogram (α and β furanosides and α and β pyranosides).

The usefulness of this procedure in LPS carbohydrate profile determination was verified using *Desulfovibrio desulfuricans* bacteria endotoxin. The 1M hydrochloride solution in methanol (derived from the reaction of methanol with acetyl chloride) was added to the freeze-dry LPS. After 24 hours of incubation at 85°C, the sample was evaporated in the stream of nitrogen, washed out with methanol and again evaporated. The derived methyl glycosides were subjected to the acetylation for 20 min at 100°C using acetic anhydride and sodium acetate as a catalyzer. Carbohydrate derivatives were dissolved in ethyl acetate and subjected to the GLC-MS.

The suggested procedure may be useful in the identification of amino sugars and some acidic derivatives as monosaccharide units of LPS complexes.

ELECTROPHORETIC STUDYING OF COMPLEXATION PROCESSES OF COPPER WITH BIOLOGICALLY ACTIVE ORGANIC LIGANDS

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The vital metals are in an organism mainly in the coordination compounds form. The deficiency or lack in such metals, intervention of toxic substances and leads to infringement of normal processes in an organism and to diseases. Therefore the understanding of a role of coordination compounds in biosystems can be a key for the creation of a new classes of effective drugs.

In a mode capillary zone electrophoresis (CZE) with UF-detecting the opportunity complexation copper with biologically active ligands was studied with 3,4-dihydroxyphenyl-alanine (DOPA), norepinephrine, glutamic acid. Thus various variants of electrophoresis control over processes complexation were realized: change molar ratio of concentration of copper ions and ligands; the introduction previously prepared complex into a quartz capillary of; the presence one of the participants of complexation in the composition of background buffer electrolit.

Three various schemes of electrophoretic analysis were realized for all investigated organic ligands with copper. The dependences of intensity of response from as ratio of molar concentration L : Cu as time of sample injection into a capillary were realized. It was shown that it is possible to control complexation prosess in the system "biological ligand - metal" using method CZE. Figure 1 presents electrophoregram of DOPA+Cu complex, figure 2 – dependence of response intensity of peaks DOPA and complex DOPA+Cu from ratio of molar concentration Cu : L.

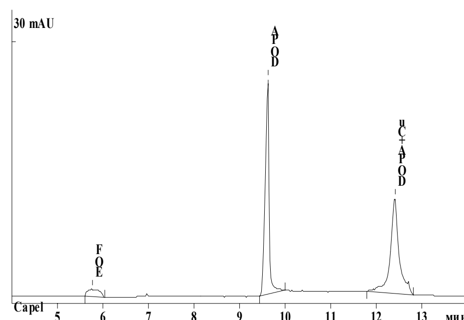

Fig. 1. Electrophoregram of complex DOPA $4x10^{-4}$ M + Cu $2x10^{-4}$ M.

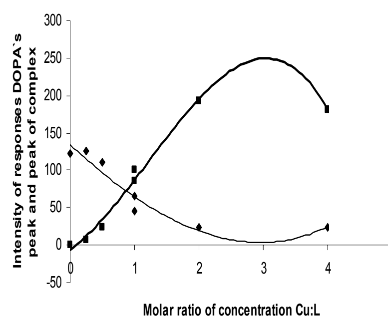

Fig.2. Dependence of response intensit of peaks DOPA (1) and complex DOPA+Cu (2) from the molar ratio of concentration Cu:DOPA.

Conditions: system of capillary electrophoresis "Capel-105 R" (Lumex Ltd., St. Petersburg) (λ = 220 nm), 35 mM borate buffer pH 8,6.

NORMAL-PHASE THIN-LAYER CHROMATOGRAPHY OF SEVERAL ANGIOTENSIN CONVERTING ENZYME INHIBITORS

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Angiotensin converting enzyme (ACE) inhibitors are considerable group of well tolerated drugs widely applied in the prevention and treatment of hypertension and congestive heart failure. On administration esterified pro-drugs are transformed into active di-acid metabo-lites. Several methods have been proposed for analysis of ACE inhibitors in pharmaceutical formulations and biological fluids, including a few planar chromatographic ones.

In our previous papers [1-3] reversed-phase chromatographic behavior (including salting-out chromatography) of certain ACE inhibitors and their metabolites was described. Within the scope of our investigations focused on the examination of the effect of the structure of biologically active substances on their retention in different TLC systems, the present work was concentrated on chromatographic behavior of nine ACE inhibitors and corresponding meta-bolites under condition of normal-phase thin–layer chromatography on silica gel.

The results obtained showed that normal-phase thin–layer chromatography appears to be suitable method for separation of ACE inhibitors from their metabolites. On the basis of these results corresponding separation mechanisms were considered.

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[2] J. Odović, B. Stojimirović, M. Aleksić, D. Milojković-Opsenica, Ž. Tešić, *J. Planar Chromatogr.* 18 (2005) 102-107
[3] J. Odović, B. Stojimirović, M. Aleksić, D. Milojković-Opsenica, Ž. Tešić, *J. Serb. Chem. Soc., in press.*

EXAMINATION OF SEVERAL ACE INHIBITORS BY REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY ON CELLULOSE

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Continuing our previous studies of angiotensin converting enzyme (ACE) inhibitors [1-3], in this work we examine chromatographic behaviour of some ACE inhibitors and their metabolites, under conditions of reversed-phase thin–layer chromatography on cellulose (Art. 5552, Merck, Germany). The substances investigated were: *Enalapril*, *Enalaprilat*, *Quinapril*, *Quinaprilat*, *Fosinopril*, *Fosinoprilat*, *Lisinopril*, *Cilazapril*, *Cilazaprilat*.

Folloving binary solvent systems were used with varying amount of organic modifier:
water – methanol (10 - 30 vol% methanol),
water – ethanol (10 – 30 vol% ethanol),
water – acetone (10 – 30 vol% acetone).

After development by the ascending technique, the detection was performed by exposing the plates to iodine vapour. All investigations were performed at room temperature(22 ± 2°C).

The linear relationship between the R_M values and concentration of organic modifier in mobile phases was obtained. From regression data of the plots, the hydrophobicity parameters R_M^o and m were determined and C_0 parameter was calculated. Chromatographically obtained hydrophobicity parameters were correlated with calculated logP values.

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AN ATTEMPT TO CONDUCT HYDROQUINONE BIOTRANSFORMATION INTO ARBUTIN IN *Hypericum perforatum* L. *IN VITRO* CULTURES: HPLC ANALYSIS

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An attempt has been made to conduct biotransformation of exogenous hydroquinone into arbutin in *Hypericum perforatum* L. (St. John's Worth) *in vitro* cultures. Arbutin, a hydroquinone β-D-glucoside, is a secondary metabolite of plant origin used for medicinal and cosmetic purposes as a urinary tract disinfectant and skin whitener.

Hypericum perforatum agitating cultures were maintained on the Linsmaier-Skoog (LS) medium with growth regulators: NAA (2 mg/l) and BAP (2 mg) under constant artificial light (900 lx). After a 2-week growth of biomass, hydroquinone was added to culture flasks at 100 mg/l. Samples were collected 24, 48, 72, 96 and 168 h after precursor supplementation. Arbutin content was determined in methanol extracts from dry biomass using RP-HPLC (RP-8).

It was shown that *Hypericum perforatum* cultures were capable of transforming exogenous hydroquinone into arbutin. Maximum product content (14.28 mg/g d.w.) was observed after 48 h, whereas process yield was the highest (58.84 %) at 168 h after hydroquinone addition. Next stage of the research is planned to optimize this process.

NP- AND RP-HPLC OF SALICYLIC COMPOUNDS FROM THE BARK OF DIFFERENT SPECIES OF THE GENUS *Salix*

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Salicin and its derivatives are the most important compounds of the genus *Salix*. They are responsible for the pharmacological activity of willow bark - herbal remedy used since centuries in the treatment of rheumatism, pain and fever. According to the European Pharmacopoeia the starting material – bark of willow - may originate from various *Salix* species, that have to contain not less than 1.5 % salicin. Until now, the salicylic alcohol derivatives: salicin, salicortin, salireposide and tremulacin were separated by means of an HPLC method on a Spherisorb ODS II column, while other compounds: 2'-acetylsalicin, 2'-O-acetylsalicortin, tremulacin besides salicin and salicortin, were resolved on a silica gel layer by HPTLC method.

The aim of our work was to optimize the HPLC separation of the mixture of salicin and other simple phenols: salicortin, salidroside, picein, helicin, triandrin, populin and tremulacin by use of RP- and NP- approaches. The NP - HPLC separation was performed on a Chromolith Performance Si (100 × 4.6mm) column with isocratic elution (mobile phase: hexane/isopropanol/methanol 87/12/1, *v/v/v*). At RP-HPLC conditions, two columns were coupled in series, namely Chromolith Performance Si and Chromolith Performance RP18e (100 × 4.6mm) (Merck, Germany). The resolution was tested with different gradient elutions: 1) increasing concentration of tetrahydrofuran from 3 to 53 % within 30 min in a mixture of water / trifluoroacetic acid (TFA) (99.95/0.05, *v/v*), and 2) increasing concentration of acetonitrile from 3 to 48% within 27 min in water / trifluoroacetic acid (TFA) (99.95/0.05 *v/v*). For the selective detection of salicylic compounds, besides a UV detector, the evaporative light scattering (ELS) detector was employed. The optimized conditions were applied to verify the presence of salicylic compounds in the methanol extracts from the bark of *Salix purpurea, S. daphnoides, S. alba, S. triandra, S. viminalis* and *S. herbacea*.

Furthermore, an SPE-HPLC method was developed for the rapid analysis of the content of salicin in analysed plant material. The highest amount of salicin was detected in the extracts from the bark of *Salix daphnoides*, both before and after alkaline hydrolysis.

Acknowledgments:

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OPTIMIZATION OF CONDITIONS FOR ISOLATION AND DETERMINATION OF BISPHENOL A IN MILK

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The recently undertaken research showed that significant part of the chemical substances perceived by the humans, such as natural and synthetic hormones, metalorganic compounds, durable organic compounds, monomers, and some chemical additions used in the plastic industry, are able to disturb the natural hormonal balance of the human body, as well as to cause several diseases both for the humans and the animals [1-3]. Particular attention has been paid recently to Bisphenol A (BPA) [4-7]. This compound is used for many industry activities, including the synthesis of polycarbonate plastics, epoxy resins, and polyacrylates. These plastic materials are massively used for the productions of (among others) baby bottles and internal coating layers for the packings for baby food industry, such as powdered milk and milk mixtures [7].

Recently, many publications confirmed estrogenic activities of BPA *in vivo*, even taken in very little doses. These are foetus and little babies that are the most sensitive group taking into account hormonal aspects of BPA exposal. It seems that the main way of BPA penetration is related with milk, both breast milk and some diet supplements (e.g., powdered milk). The poster presents our research concerning determination of BPA in the milk and milk-derivative products. The main goal was to optimize the conditions for the isolation (by the use of SPE) and determination of BPA in the milk samples, by the use of gas chromatography coupled with Flame Ionization Detection (FID), and Low Resolution Mass Spectrometry (LRMS).

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ON-LINE SILYLATION IN DETERMINATION OF NONSTEROIDAL ANTIINFLAMMATORY DRUGS

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Some nonsteroidal antiinflammatory drugs (NSAIDs), such as naproxen, ibuprofen, diclofenac and ketoprofen, are classified as "over the counter" analgetic agents. Due to facilitated availability, the risk of their prolonged abuse or accidental overdose by self-treated patients is relatively high.

Unlike antihypertensive or antidiabetic drugs that cause easily detectable changes in some physiological or biochemical parameters (blood pressure, sugar level), the effects of NSAIDs are not so characteristic and their quantitative evaluation is difficult. Thus, for toxico-logical reasons, it is necessary to elaborate a rapid, simple and accurate method for determi-nation of individual NSAID concentration in biological fluids, including full blood.

In the present study, the authors have developed the new technique of naproxen, ibuprofen, diclofenac and ketoprofen determination by on-line derivatization. NSAID-containing extract of the sample was mixed with hexadimethylsilazane (HMDS) and introduced into the hot split/splitless injector. Silyl derivatives formed under such conditions were then analyzed by GC/MS.

All the parameters of the derivatization process (including thermochemolysis temperature) and subsequent chromatographic separation have been optimized. Dichloromethane was chosen as the most effective extraction solvent.

As compared to conventional derivatization techniques, the yield of on-line silylation of the studied NSAIDs is very high (above 90%). Furthermore, the described method is rapid and less laborious, and allows reducing amount of the sample required for a single analysis.

It was concluded that the developed on-line silylation method could be applied for qualitative and quantitative analysis of naproxen, ibuprofen diclofenac and ketoprofen in biological samples.

THE APPLICATION OF MICELLAR MOBILE PHASES FOR THE CORTICOSTEROIDS SEPARATION BY HIGH PERFORMANCE THIN-LAYER CHROMATOGRAPHY (HPTLC) AND MICELLAR ELECTROKINETIC CHROMATOGRAPHY (MEKC)

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The addition of micelles into the buffer solution (the method of MEKC) or into the eluent (the method of HPTLC) allows the separation and concurrent determination of endo- (cortisol, cortisone, corticosterone, 11-deoxycorticosterone) and exogenic steroids (prednisolone, cortisone acetate, dexamethasone, cortineff).

MEKC allows the separation of neutral and ionic analytes. The sodium dodecyl sulphate was brought in the running buffer in concentration more then critical concentration of micelleformation. It reduces to increasing the separation selectivity. Micelle is «*pseudo-stationary phase*» and analytes divides between micelle and running buffer. Organic solute (urea) brought in the running buffer decreases the hydrophobic interactions between analytes and micelles and influenses on the electrophoretical mobility of analytes (Fig. 1).

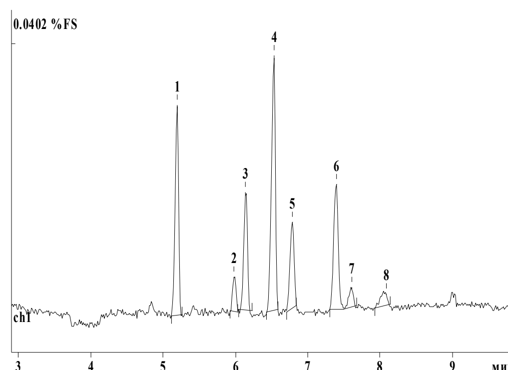

Figure 1. Electrophoregram of steroid model sample, matrix of sample – water, injection 60 mbar. Sample - 1 - 11-deoxycorticosterone (10 μg/ml); 2 – dexamethasone (10 μg/ml); 3 - cortisone acetate (10 μg/ml); 4 – corticosterone (10 μg/ml); 5 – cortineff (10 μg/ml); 6 – prednisolone (10 μg/ml); 7 – cortisone (1 μg/ml); 8 – cortisol (1 μg/ml). Conditions: «Capel 103P» (λ_{max} = 254 nm), operating voltage was -25 kV, current 96 μA. Buffer: 25 mM phosphoric acid, 20 mM SDS, 4,5 M urea, pH 2,5. Fused silica capillaries of 60 cm (50 cm to detector)x75 μm I.D.

The HPTLC are enough convenient for laboratory express diagnosis. We perfomed the concurrent separation of exo- (drugs: prednisolone, cortisone acetate, dexamethasone) and endogenic corticosteroids (cortisol, cortisone) using HPTLC with UV and videodensimetric detection (λ = 254 nm). The optimal mobile phase was the water solution with the addition of 12 mM sodium dodecylsulfate.

The dependences of retention factors for prednisolone, cortisone and cortisol from the contents of SDS are linear (the dates are in Fig. 2). For the rest of steroids (dexamethasone and cortisone-acetate) linear dependence are only in the concentration area of 8,3 – 20 mM SDS.

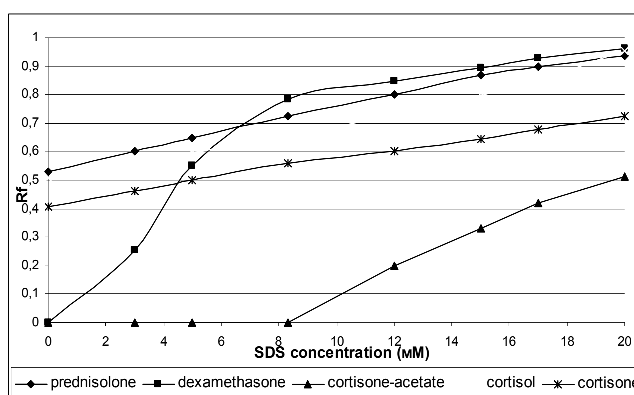

Figure 2. The dependence of R_f steroids from the contents of SDS in the mobile phase.

So the application of micellar mobile phases allows the increasing of selectivity of MHPTLC and MEKC caused by the presence of SDS micelles in the eluent.

USING MICELLAR LIQUID CHROMATOGRAPHY FOR ANALYSIS OF BISPHENOL A

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There are a number of chemical substances that disturb regular performance of the hormonal system. They are referred to as endocrine disrupters and their undesirable effect is felt by both men and women. These substances disturb the hormonal equilibrium of organisms, which is particularly dangerous in developmental age, when the changes are in most of the cases, irreversible. Destabilisation of the hormonal system can lead to a number of physiological effects.

The endocrine disrupting compounds EDCs are divided into three groups: pharma-ceutical, natural, and some environmental pollutants. The compounds present in the natural environment and showing estrogenic properties are the following (among others): organochlorine pesticides, alkylphenols, phthalates, polychlorinated biphenyls, dioxins and bisphenol A.

Bisphenol A (BPA) has been used as a raw substance for mass production of. epoxy resin, polycarbonate, polyester and polyacrylate plastics. BPA been used as a fungicide, antioxidant, an agent suppressing inflammability in rubber industry, and plastic production, and as a stabiliser in production of polyvinyl chloride. BPA and its derivatives are potentially hazardous to the consumer health thus their presence and concentration level in food products should be monitored.

Polycarbonate plastics (PC) are commonly used for production of food product packages, bottles for water, bottles for infant food, kitchen utensils, and some elements of medical equipment. The PC-made substances are able to release BPA, when used for coverage of inner surfaces of tins for food products or in some dental fillings. BPA residues have been found in the water and other food products stored in PC packages. BPA is able to liberate from a PC package and migrate into the food stored inside. Such a migration is favoured by acidity of the product stored, elevated temperature, mechanical cleaning and the use of detergents for cleaning bottles or other PC packages.

The analysis of BPA has been accomplished by HPLC, GC and MEKC.

In our work, high performance liquid chromatography with micellar mobile phase (MLC) was used for the determination of bisphenol A.

DERIVATIZATION AND GAS CHROMATOGRAPHY-LOW RESOLUTION MASS SPECTROMETRY OF BISPHENOL A

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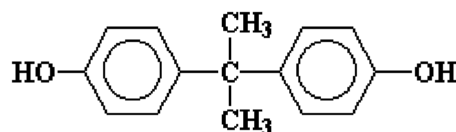
Bisphenol A (BPA) is a known environmental estrogen that is used as the monomer to manufacture polycarbonate plastic, the resin that is used as linings for most food and beverage cans, as dental sealants, and as an additive in other widely used consumer products. The level of human exposure to BPA is not insignificant, as microgram amounts of BPA were reported to be detectable in liquid from canned vegetables. Recently, a large number of *in vitro* studies show that estrogenic effects of BPA occurring at doses as low as 1 pM or 0.23 ppt [1]. Therefore, sensitive analytical methods are required to identify and determine trace levels of this compound both in environmental and biological matrices.



bisphenol A

The aim of our study was to evaluate the method of BPA derivatization, for increase a limit of detection and determination of this compound by gas chromatography-low resolution mass spectrometry (GC-LRMS). Therefore, standard solutions of BPA and milk extracts were derivatized in reaction vials with three different derivatization agents: – N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) with 1% of trimethylchlorosilane (TMSC); bromoacetonitryle and trifluoroacetic anhydride [2-4]. All GC experiments were carried out with fused silica capillary column PE-5MS®, Perkin-Elmer, USA (20m, 0.18 mm I.D., 0.18 µm film thickness; 5% diphenyl - 95% dimethyl siloxane) installed in Autosystem XL chromatograph (Perkin Elmer, USA) equipped with autosampler, split-splitless injector and Turbomass (LRMS) detector.

Acknowledgement:

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THE ANALYSIS OF METABOLITES PRODUCED BY ENTOMOPATHOGENIC FUNGUS *Conidiobolus coronatus*

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Entomopathogenic fungi are promising tools as biological agents of insect pests. New insecticides which act specific without contamination of the environment are desirable. Several fungal metabolites toxic to insects were isolated and characterised, but in Entomopthorales (Zygomycota) the knowledge of theirs toxins is not well recognised.

In our laboratory we work on entomopathogenic fungus *Conidiobolus coronatus*. It is a pathogenic fungus which kills host insects rapidly. The fungal invasion occurs through penetration of the host integument. Death of the host occurs as a result of the production of toxins by this fungus. We tend to isolate and characterise toxic proteinaceus compounds, which my lead to the creation of new generation of safe and selective insecticides.

With the use of HPLC fungal mycelial homogenates are fractionated. The toxicity of separated fractions is estimated by injections into hemocoel of *Galleria mellonella* larvae. The enzymatic activity of fractions are measured with the use of synthetic substrates suitable for proteinase, chitinase and lipase assessments. The purity of fractions are checked with SDS-PAGE.

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SEPARATION OF ERGOSTEROL AND RELATED STEROIDS UNDER TEMPERATURE CONTROLLED TLC CONDITIONS

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The aim of this work was to study the influence of temperature on retention of selected compounds using non-forced planar chromatography in normal and reversed phase mode. As the components of interest ergosterol and number of related steroids that may be considered as the environmental biomarkers of the endocrine disrupting phenomenon including stigmasterol, cholesterol, dihydrocholesterol, 4-cholesten-3-one, 7-dehydrocholesterol and cholesterol acetate were selected. Moreover retention of 7,8-dimethoxyflavone was also studied due to broad application of such substance as the internal standard frequently used for quantitative analysis of steroids.

Chromatographic plates (K60WF_{254}S and RP18W) were developed using mobile phases composed of methanol-dichloromethane and methanol-water mixtures. Separation process was performed in commercially available horizontal chamber Chromdes DS-L (Lublin, Poland). In order to ensure given sub-ambient or elevated temperature, chromatographic chamber was placed in the thermostatically controlled oven, which was operating for temperatures ranging from 5 to 55 ^{o}C. In contrary to the retention data obtained previously for bile acids [1], the results of present study revealed relatively low temperature-retention response of the components of interest, particularly, for separation performed on silica plates and methanol-dichloromethane mobile phase.

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VISUALISATION PROTOCOLS FOR PLANAR CHROMATOGRAPHY USED BY THE EUROPEAN PHARMACOPOEIA

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Planar chromatography is still commonly used as the simple and efficient tool for pre-purification, separation, detection and quantification of number of components of interest that are present in complex pharmaceutical, biological and environmental samples. Unlike column chromatography, the planar counterpart can easily work as a two-dimensional method allowing efficient and fast separation of analytes from complex biosamples including blood, urine, tissue, sewage water or sediments. The advantage of TLC detection is that the bands or spots developed can be easily inspected under visible and UV light (usually 254/365nm) and photographed using digital cameras without use of expensive scanners devices. Thin layer chromatography is frequently applied for sensitive detection of UV transparent compounds from pharmaceutical formulations using number of simple staining reagents including iodine vapour, vanillin, ninhydrin, thymol, dichlorofluorescein, antimony chloride, ferric chloride, dinitrophenylhydrazine, anisaldehyde, potassium iodobismuthate, ferricyanide, dichromate, permanganate or hydroxide as well as phosphomolybdic, nitric or sulphuric acid. The main goal of this work is to discuss the contribution of TLC detection methods for quantification, purity confirmation and substance identification protocols described in the European Pharmacopoeia monographs [1]. Moreover, the comparison of TLC and HPLC detection for particular class of compounds as well as critical review of the visualisation protocols based on *e.g.* phosphomolybdic acid staining methodology will be discussed [2,3].

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APPLICATION OF HPLC TO THE INVESTIGATION OF CHRONIC ORAL LEVODOPA/CARBIDOPA THERAPY ON PATIENTS WITH PARKINSON'S DISEASE

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Parkinson Disease (PD) is relatively often appearing illness – especially of elder people. Its influence on quality of life is significant; PD symptoms like tremor or "off" state are very uncomfortable for patients who are seeking methods of avoiding these troubles.

The aim of the study was to investigate correlation between levodopa concentration, Webster test results and tremor – time profiles following the oral dose of 200 mg levodopa. Up till now different components of PD – physiological, mechanical and psychological – were treated separately, what was causing that these different aspects of PD couldn't be compared. Such multidimensional approach to PD is original and the task was to give the physician new tools for better medication of this illness.

Tree mentioned above methods of PD symptoms intensity measurement were applied. First of them is chromatographic measurement of levodopa concentration. It was performed by means of a chromatograph comprising a Interface Box, 4 channel Degasser K-5004, Solvent Organizer K-1500, Dynamic Mixing Chamber, HPLC Pump K-1001, Fast Scanning UV Detector K-2600, Eurochrom 2000 chromatographic data acquisition and analysis software (all from Knauer GmbH, Berlin, Germany) Basic$^+$ marathon.

Original tremor measurements assay and procedure have been elaborated in the Department of Electronics (Military University of Technology) in co-operation with the Neurological Clinic Department of Neurology (Military Institute of Medicine). The assay consists of two separate accelerometers, with frequency band from 0.1 to 16 500 Hz, for the left and right hand, signal amplifiers, A/D converter and personal computer with the software. Transmission band has been restricted to the interval <2; 20> Hz with respect to defined earlier real interval of limb tremor. As input data acceleration of hand movements was registered (precisely measured at thumb of both hands). Sampling period 80 s at frequency 100 Hz gives 8192 samples for one measurement.

Webster test was performed by means of special questionnaire adapted for the task. Ten parameters were estimated by patient on four – grade scale (0 – 3) where 0 means no symptoms and 3 – great intensity of symptom, causing fundamental difficulties in everyday living. Sum of this ten valuations (varying from 0 to 30) determined final test value.

All these measurement were performed 5 times daily – immediately before first levodopa ordination, and following after every hour. The temporary profiles of some aspect of PD symptoms were obtained in this way.

Obtained results show, that there is significant correlation between these three measurements – especially between levodopa concentration and tremor level. The measurement of levodopa concentration is much more complicated and lasting then tremor measurement, so it allows for better treatment of patients.

DETERMINATION OF HYDROXYUREA AND (*RS*)-*N*-[(1-ETHYLPYRROLIDIN-2-YL)METHYL]-2-METOXY-5-SULPHAMOYLBENZAMIDE IN THE WORKPLACE AIR IN PHARMACEUTICAL INDUSTRY BY HPLC

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In the past several years, one of the primary tasks of environmental chemistry has been the detection, determination and fate studies of pharmaceuticals in different compartments of environment, particularly in water ecosystems. Following the pathways of pharmaceuticals in the environment we should also consider the pharmaceutical manufacturing operations. Workers involved in the manufacture of drug substances are exposed to the active pharmaceutical ingredients which can interact with the human system and modificate of its functioning. Apart from pure active pharmaceutical form this exposure involves also chemicals such as solvents, fillers, flavouring and bulking agents, and it occurs by inhalation of drug dust or aerosol, absorption through the skin, contact with contaminated clothes and other sources. Workers involved in the synthesis these compounds need to be protected from exposure. One approach is establishing of an acceptable airborne level for such compound in the workplace. Suitably validated analytical methods need to be develop to achieve required sensitivity.

The objective of this study was both a method development for determination of N-hydroxyurea and N-[(1-ethylpyrrolidin-2-yl)methyl]-2-metoxy-5-sulphamoylbenzamide in air sample in the worklpace.

N-hydroxyurea is an antineoplastic drug used in hematological malignances. Method of determination involves adsorption on the fibrous glass filter, single extraction procedure and chromatographic analysis. The mobile phase consists of water and methanol (95:5). The absorbance of eluent was monitored at λ=214 nm using DAD detector. The limit of detection was 0.069 µg/ml and recovery coefficient of hydroxyurea was 98%.

N-[(1-ethylpyrrolidin-2-yl)methyl]-2-metoxy-5-sulphamoylbenzamide is used in treatment of depression, acute psychosis, obsessional neurosis and behavioral disorders. Method of determination involves adsorption on the fibrous glass filter, single extraction procedure using methanol and HPLC analysis. Chromatography was performed at 35 °C using a mobile phase consisting of methanol – phosphate buffer with pH 3.0 (16:84). These measurements were done with DAD (240 nm= and FLD (ex.300 nm; em. 365 nm). The detection limit was 0.084 µg/ml and 0.0033 µg/ml, respectively. The recovery coefficient was 83%.

In order to verify the developed methods measurements were also taken in the industrial condition.

DETERMINATION OF IMIDACHLORPRIDE IN THE CHESTNUT TREE LEAVES

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Imidachlorpride belongs to the group of chloronicotiniles and presently is considered as the most popular systemic insecticide.

It was the aim of this study to elaborate the method of imidachlorpride quantification in chestnut tree leaves, when applying the imidachlorpride preparation directly to the chestnut tree trunk in order to protect it against the *Cameraria ohridella* (the family of *Gracillariidae*) isect, currently the most dangerous destroyer of chestnut trees.

A sample of lyophilized leaves was extracted with aid of dichloromethane with use of ultrasonic bath. Solvent was removed from the extract with use of evaporator and the dry residue was dissolved in 2 mL ethyl acetate. The ethyl acetate solution was introduced on top of the chromatographic column filled with 10 g Florisil. Then the column was eluted with 100 mL ethyl acetate and 25 mL acetonitrile in order to wash out the adsorbed pesticide. The eluate was evaporated to dryness and dissolved in 2 mL of the acetonitrile/water (1:1, *v/v*) liquid binary mixture.

The prepared sample was analyzed by means of HPLC/DAD. An additional effort was also undertaken to make use of thin layer chromatography coupled with densitometric detection in order to separate the extract and quantify imidachlorpride contained therein.

MESUREMENTS OF CYCLOPENTANE AND CO_2 EMISSIONS FROM POLYURETHANE FOAM BY GC-MS

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Polyurethane (PUR) foams are one of the most widespread thermal insulation materials in the world. PUR foams release to ambient carbon dioxide, cyclopentane or soft freons – gases closed during manufacturing inside polymer cells due to improve its thermal resistance. Emitted gases were first quality analyzed using headspace sampler (HP 7694E) and gas chromatograph (HP 6890) coupled with mass quadrupole detector (HP 5973). Emission tests were developed using hermetical chamber direct connected to gas chromatograph. Carrier gas flowing through the chamber flushed of compounds emitted from foam surface to sample loop. Carrier gas flow was set to achieve 1 and 10 gas change in the chamber per hour. Measurements were carried out at 23, 40, 60 and 80 °C. Calibration were performed in the same test chamber at 80 °C using exponential dilution method [1]. Initial concentrations of cyclopentane and CO_2 in foam cells were determined by grinding of cylindrical samples (squeezing of gases from cells) in crusher [2] connected to gas chromatograph through the sample loop. From emission and cell gas composition data effective diffusion coefficients basing on [3] and activation energies of diffusion were calculated for both values of gas change in the chamber.

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A COMPARISON OF ANALYTICAL METHODS FOR DETERMINATION OF MINERAL OIL IN ENVIRONMENTAL SAMPLES

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Mineral oil analyses are frequently used for the goal of finding areas of gross contamination. If mineral oil concentrations are very high, it usually signifies that significant amounts of contaminations are there. Mineral oil is often referred to as total petroleum hydrocarbons - *TPH*, hydrocarbon oil, extractable petroleum hydrocarbons, and oil and grease. Mineral oil is defined as the measurable amount of aliphatic petroleum-based hydrocarbon in an environmental media and represents a summarize parameter rather than an ensemble of individual compounds. There are many analytical techniques available that measure mineral oil concentrations in the environment. The definition depends on the analytical method used because the analytes measurement is the total concentration of the hydrocarbons extracted and measured by a particular method. The same sample analyzed by different methods may produce different concentration values. For this reason, it is important to know exactly how each determination is made. Interpretation of the results depends on understanding the capabilities and limitations of the selected method. In this work different analytical methods for isolation and determination of mineral oil were studied. Preconcentration techniques (such as: Soxhlet extraction, Accelerated Solvent Extraction - *ASE*, ultrasonic and liquid extraction) for isolation of mineral oil from environmental samples (soil, sediments) were examined. Different quantitation techniques (such as: gas chromatography – flame ionization detection (*GC-FID*), infrared spectroscopy (*FTIR*), gravimetric methods and *TPH* - rapid assay) were used.

BIODEGRADATION OF BROWN COALS CAUSED BY FUNGI AND BACTERIA

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Biodegradation is one of the most important processes changing composition of bituminous fraction of organic matter and affecting results of geochemical investigations [1]. The process was extensively investigated for crude oils since it affects oil quality, however in the case of brown and bituminous coals only limited number of research was done [2].

To assess the influence of the process in selected brown coals the powdered samples were extracted in DCM to receive bituminous fractions. The extracts were separated into aliphatic, aromatic and polar compounds by preparative layer chromatography (Kiesegel 60, Merck). Plates were developed in *n*-hexane. Aliphatic and aromatic hydrocarbon fractions were investigated by GC-MS with Agilent Techn. gas chromatograph with HP-5 column (60m x 0.25mm), coated by 0.25 μm stationary phase film was applied. The gas chromatograph was coupled to a mass spectrometer operating in EI mode at 70 eV (full scan, 50 - 650 da). Data were processed with the Hewlett Packard Chemstation software. All compounds were identified by their mass spectra, comparison of retention times to these of standard compounds and literature data and interpretation of MS fragmentation patterns [3-4].

It was found that biodegradation of organic matter of the investigated brown coals could be performed both by bacteria or fungi. Both bulk group composition of the extracts and the occurrence of particular compound groups may be affected by the process, however in many cases the changes are similar to these caused by water washing. Due to the occurrence of characteristic phenolic products being monomers of macromolecule lignin it was possible to confirm the partial degradation of this resistant component of sedimentary organic matter. It seems xylithic brown coals show higher resistance to biodegradation than detritic brown coals, probably due to the diterpenoids presence in wood tissue. These compounds having antifungal and anti-bacterial properties can partially protect lithotypes rich in them. Since detritic brown coals originate from primal organic matter deriving from grasses [5] poor in diterpenoids they are more susceptible to bacterial of fungal attack than xylithic brown coals.

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PLC AND GC-MS IN ASSESSMENT OF CHANGES IN CHEMICAL COMPOSITION OF BROWN COAL EXTRACTS CAUSED BY WATER WASHING

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Water washing affects the composition of low-molecular fraction of sedimentary organic matter by removing its lighter and more soluble components [1]. Since both the mineral matrix and macromolecular fraction of organic matter can influence the process by their variable composition it is important to find means to assess a range of alterations caused by the process.

Powdered brown coals were extracted with CH_2Cl_2. To estimate group composition of brown coal bitumen the extracts were separated into aliphatic, aromatic and polar compounds by preparative layer chromatography (Kieselgel 60, Merck). Aliphatic and aromatic hydrocarbon fractions were investigated by GC-MS with Agilent Techn. gas chromatograph equipped with HP-5 column (60m x 0.25mm), coated by 0.25 μm stationary phase film. The gas chromatograph was coupled to a mass spectrometer operating in EI mode at 70 eV (full scan, 50 - 650 da). Data were processed with the Hewlett Packard Chemstation software. All compounds were identified by their mass spectra, comparison of retention times to these of standard compounds and literature data and interpretation of MS fragmentation patterns [1-3].

Several geochemical parameters were proposed to assess extend of water washing alterations in sedimentary organic material of brown coals. In a bulk analysis water washing may be indicated by the decrease in brown coal extractability and aliphatic and/or aromatic hydrocarbon fraction content in the extracts. GC-MS shows the major changes in chemical composition of the extracts such as the progressive removal of lighter *n*-alkanes in the range *n*-C_{12}-*n*-C_{22} together with pristane and phytane, alkylnathphalenes starting from metylnaphthalenes and unsubstituted polycyclic aromatic hydrocarbons with 2-3 aromatic rings. The changes are characteristic and their extent can be measured with proposed parameters. Basing on the above findings the 10-point scale of water washing to estimate a range of alterations was proposed.

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GC-MS IN INVESTIGATION OF ORGANIC MATTER PRESENT IN ALLUVIAL AND GLACIAL SEDIMENTS

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Organic matter present in fourteen alluvial and fluvoglacial sediments of the Quaternary and Miocene age sampled in two boreholes were the object of geochemical analyses. The host sediments were extracted with DCM:EtOH (4:1; v:v) to yield low-molecular organic matter. Since very low extractability, chemical composition of the whole extracts, without group separation was investigated with gas chomatography-mass spectrometry (GC-MS). An Agilent Techn. gas chromatograph was equipped with HP-5 column (60m x 0.20 mm), coated by 0.25 μm stationary phase film. The gas chromatograph was coupled to a mass spectrometer operating in EI mode at 70 eV (full scan, 50 - 650 da). Data were processed with the Hewlett Packard Chemstation software. All compounds were identified by their mass spectra, comparison of retention times to these of standard compounds and literature data and interpretation of MS fragmentation patterns [1-2].

The GC-MS results were applied to estimate biological origin, thermal maturity and possible sources of re-worked and re-deposited sedimentary organic matter using distribution of biomarkers. It was found that there are essentially two sources of organic material present in the investigated sediments.

I. Organic matter of relatively low thermal maturity being at early diagenesis or medium advanceg diagenesis; it may be assumed this organic matter comes from fairly recent biological sources since it contains functionalised polar compounds such as sterols, limonene, ferruginol, lighter carboxylic acids or hydroacids and phenolic compounds. Some of these compound may be found in the Tertiary brown coals (vitrinite reflectance ~0,25-0,30%).

II. Organic matter of more advanced maturity being at the stage of moderately advanced catagenesis at least; the values of biomarker parameters and indices testify maturity level corresponding to vitrinite reflectance in the range R_r = ~0,60-75%. Such high maturity indicates organic matter re-deposited as a result of glacial erosion and transportation.

In most cases organic matter seems to be a mixture containing compounds deriving from both these sources; however the Miocene clay contains autochthonous organic matter only.

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GAS CHROMATOGRAPHY-MASS SPECTROMETRY IN INVESTIGATION OF FIRES IN COAL WASTE DUMPS

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Coal mining industry is one of major waste producers in Poland. Annually, it produces about 0.6-0.7 ton of minestone per ton of exploited coal, next collected in 136 waste dumps. Such coal wastes, containing relatively high amounts of organic material (on average 7-15%) are susceptible to heating and fire spontaneously sometimes occurring in them.

Twelve samples of coal waste affected by fire to the various extend were collected from the coal waste dump in Piekary Śląskie (Upper Silesia, Poland) sampling them at various distance (from 0,05 to >4m) from the heat source to show different degree of transformation. Powdered waste samples were extracted with a mixture of DCM:EtOH (4:1, v:v). The composition of total extracts was investigated with GC-MS using an Agilent Techn. gas chromatograph equipped with DB-35 column (60m x 0.25mm), coated by 0.25 µm stationary phase film. The gas chromatograph was coupled to a mass spectrometer operating in EI mode at 70 eV (full scan, 50 - 650 da). Data were processed with the Hewlett Packard Chemstation software. All compounds were identified by their mass spectra, comparison of retention times to these of standard compounds and literature data and interpretation of MS fragmentation patterns [1-2].

GC-MS analysis shows that waste extracts were affected by fire in a various degree. Some of them show features typical for low-temperature coal tars, in the others organic matter was almost completely destroyed, while one of the samples has features of the original coal. Less thermally stable compounds, such as lighter *n*-alkanes, cyclic isoprenoids, methyl and dimethylnaphthalenes, methyphenanthrenes and five-ring polycyclic aromatic hydrocarbons were destroyed or evaporated in the case of samples which were more affected by temperature. To assess the extent of thermal changes in OM caused by heat several thermal maturity biomarker parameters were calculated based on aliphatic and aromatic hydrocarbons [2-3]. Variability of their values is high, from the samples relatively thermally unaltered, which parameter values indicate beginning of catagenesis, up to the samples which maturity corresponds to the beginning of methagenesis.

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CHROMATOGRAPHIC CHARCTERIZATION OF ORGANIC MATTER PRESENT IN GLACIAL SEDIMENTS AND MELTWATER (SPITSBERGEN)

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Sedimentary organic matter dispersed in glacial sediments and meltwater from Spitsbergen was the object of chromatographic analyses. The host rocks were extracted with CH_2Cl_2 in an ultrasonic bath to yield low-molecular organic matter and meltwater samples were the object of SPE extraction (BAKERBOND C18 speed discs). Composition of extracts was investigated with GC-MS. An Agilent Techn. gas chromatograph was equipped with HP-17 column (60m x 0.25mm), coated by 0.25 µm stationary phase film. The gas chromatograph was coupled to a mass spectrometer operating in EI mode at 70 eV (full scan, 50 - 650 da). Data were processed with the Hewlett Packard Chemstation software. All compounds were identified by their mass spectra, comparison of retention times to these of standard compounds and literature data and interpretation of MS fragmentation patterns [1-2].

Several geochemical parameters were calculated to assess biological origin of organic matter, its stage of water washing and thermal maturity [2]. It was found that water washing affected only limited number of organic matter found in glacial meltwaters and concerns mainly lighter *n*-alkanes and methylnaphthalenes. The discrepancies in values of thermal maturity indices and the presence of biochemical precursors and their geochemical products (e.g. steranes and sterols, hopanes and moretanes) testify that there are two main types of organic matter in the investigated samples, both sediments and waters:

1) organic matter of relatively low thermal maturity being at early diagenesis; it may be assumed this organic matter comes from tundra vascular plants recent or nearly recent age;

2) organic matter of more advanced maturity, different in sediments from different parts of the glacier, however higher than R_r = 0,50% (the beginning of catagenesis) in all samples. Its biological origin is variable, possibly algal with significant vascular land plants input [2]. Conifers did not participate in formation of the primary organic material in this case since only low concentrations of diterpenes occur.

It may be assumed that organic matter present in meltwater does not come from the same host rocks and glacial sediments due to its much higher thermal maturity and different distribution of biomarkers investigated.

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APPLICATION OF THIN-LAYER CHROMATOGRAPHY AND SPECTROPHOTOMETRY IN ANALYSIS OF SULPHUR-CONTAINING AMINO ACIDS

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Sulphur-containing amino acids (methionine, cysteine, cystine) play an important role in many biological processes. They participate in a variety of cellural functions, among other protein synthesis, detoxification and metabolism. They are complements for formation, growth and maintenance of hair and nails. L-cysteine, as a thiol-containing substance, is considered as antioxidant.

The aim of this work was to elaborate the conditions of analytical investigations for determination of sulphur-containing amino acids in complex pharmaceutical preparations. This work is a continuation of a study carried out in Department of Analytical Chemistry of Silesian University [1].

The steps of analytical procedure were as follows:

- preparation of drug to analysis (grinding, dissolution in water)
- thin-layer chromatographic separation of amino acids of drug solution
- detection of amino acids
- extraction of sulphur-containing amino acids from the chromatogram
- spectrophotometric determination of this compounds in obtained extracts

The spectrophotometric quantification of sulphur-containing amino acids was based on the redox reactions proceeding in the system iodate ions, amino acid and leuco xylene cyanol FF. The absorbance of xylene cyanol FF dye formed was measured at 613 nm. The procedure has been applied to the determination of mentioned amino acids in selected pharmaceutical preparation.

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FOSSIL FUEL BIOMARKERS IN UBRAN AEROSOLS OF KRAKÓW, POLAND

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The particulate matter samples collected from autumn 2003 to summer 2004, at three sampling sites in Krakow were analysed using GC/MS to investigate the characteristics of selected molecular markers in urban aerosols. These compounds, relatively stable in in the atmosphere were utilized to indicate the dominant sources of anthropogenic emmision.

The concentrations of triterpenoids: hopanes, steranes and diasteranes were estimated and their distribution profiles were determined. The presence of hopanes in atmospheric aerosol indicates petroleum residues and pyrogenic components from processes of fossil fuels combustion. Their distribution profile is chracteristic for particular fossil fuel used for combusion purposes, therefore it is very useful in source indentification of anthropogenic pollutants. Steranes and diasteranes are stable constituents of vehicular engine lubricants and their presence in urban aerosol indicates the emission from traffic sources.

Moreover, isoprenoid hydrocarbons including pristane and phytane were analysed in aerosol samples. These biomarkers indicate a direct input of petroleum fuel from vehicular engines.

STUDY OF THE HEXABROMOCYCLODODECANE (HBCD) DETERMINATION IN FOOD AND THE ENVIRONMENT

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Over the last years has shown a big interest to brominated flame retardant (BFRs). The three most popular and frequently analyzed BFRs are polibrominated diphenylethers (PBDEs), hexabromocyclododecane (HBCD) and tetrabromobisphenol-A (TBBP-A). Different GC/MS methods for PBDEs analysis are available now. As about HBCD, DecaBDE and TBBP-A the analytical methods are being developed.

HBCD attract our attention in actual work. Hexabromocyclododecane (HBCD) is a crucial additive flame retardant used in various flame resistant polystyrene and textile production. The total consumption of HBCD in European Union is about 10,000 tons per year. Compared to other flame retardants only a few data on HBCD levels in the Environment have been published.

HBCD consists of three diastereoisomers – α, β, and γ. For the separation of this three HBCD isomers liquid chromatography coupled to mass spectrometry (LC/MC) is commonly used, however, GC/MS has a higher detectability, but only provides information about the total HBCD concentration in sample.

In our study we used the technical mixture of HBCD containing of 95% 1,2,5,6,9,10-Hexabromocyclododecane (1,2,5,6,9,10-HBCD). All analyses was performed using GC/ECD equipped with CP SIL 5CB GC Capillary Column of 30m x 0,32mm DF=0,25µm.

We also used semipermiable membranes (SPM) for HBCD separation from matrix compounds. Efficiency of this method for sample clean-up is enough for its determination. The recovery of HBCD is on the level of 62-101%. Therefore, using of SPM seems to be suitable clean-up method for the determination of HBCD in food and environmental samples.

Because of the thermal liability of HBCD diastereoisomers gas chromatographic separation should be performed in the temperature lower than 160^0C. At the temperature of about 160^0C thermal isomerisation of HBCD is observed. Despite this phenomena, gas chromatographic separation carried out in the temperature range up-to 250^0C caused no problem with total HBCD detection.

The work was supported by the International Visegrad Fund (US-063-2005).

SORPTIVE EXTRACTION OF ACYL XENOESTROGENS DERIVATIVES FROM WATER AND THEIR IDENTIFICATION

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Natural estrogen, 17-beta-estradiol, its oxidation product – estron, and contraceptive additive 17-alfa-ethynylestradiol are xenoestrogens which can be found in the environment and which pollute mainly water environment. They have been drawing the scientists' attention for almost few years due to their significant biological activity.

These compounds belong to the group of pollutants called endocrine disrupters (EDs). They are present in water environment in spite of biodegradation processes that take place in water.

Silicone rods with diameter of 2 mm and 20 mm long were applied to extract acyl derivatives of some xenoestrogens from water for the screening of highly contaminated water samples. Xenoestrogens reaction into acyl derivatives was performed in situ in water, and then the rods were placed in a vial for extraction of analytes. After the extraction the rods were placed into 250 μl inserts of 2ml vials filled with 100 μl of CH_2Cl_2. Desorption was performed with sonification. Qualitative composition of the extract was analysed by GC-MS.

Our work presented here includes preliminary investigations on the optimization of the derivatives' sorption on the standard water samples with the use of gas chromatography with FID detector. A solvent for desorption was selected and the following were determined: relation between sorption efficiency and sorption duration, time of desorption from extracting material and losses in preconcentration stage.

DETERMINATION OF ACYLGLYCEROLS IN DIESEL OILS BY GAS CHROMATOGRAPHY – VALIDATION OF THE METHOD

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Production of biofuels has become of vital importance in view of the dynamic development of transportation practically in 100% dependent of petrol and being the greatest local and global pollutant. Realisation of this situation has been reflected in the Directive of the EU Parliament 2003/30/EC of 08.05.2003, which obliges the EU member states to introduce biofuels to transportation in the amounts of 2% till 2005, 5.75% till 2010 and 20% till 2020.

Evaluation of the use of esters produced from vegetable oil (FAME) as biofuels requires determination of the content of mono-, di- and triacylglycerols, free and total glycerol. The contents of these compounds determine the quality of a given product and a possibility of its use as fuel or fuel component. The procedure of determination of glycerine and acylglycerols in the products of transesterification is given in the norm EN 14105. However, this procedure applies to FAME only and can be performed prior to mixing with diesel oil. Although some experimental methods for estimation of the quality of mixtures of FAME and diesel oil have been proposed, they do not permit a determination of acylglycerol content. The new analytical method proposed permits determination of acylglycerols in a diesel oil containing biocomponents.

The method has been tested on the oil samples of B5, B10, B20 and B50, and validated for FAME obtained in transesterification of rapeseed oil.

IDENTIFICATION OF INDIVIDUAL POLYCHLORINATED BIPHENYLS IN SEDIMENT EXTRACTS USING CHROMATOGRAPHIC DATABASES

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Chromatographic databases reported in literature [1-3] were applied for correlation analysis to identify individual congeners of polychlorinated biphenyls (PCBs) in two different environ-mental extracts (river sediment and sewage sludge). The retention indices of all 209 congeners were predicted using: modified Chu's equation [3,4], based on the experimental relative retention times of the seven PCBs reference series (PCB IUPAC Nos 18, 52, 101, 149, 185, 203 and 206) that exhibit linear relative retention time behaviour as a function of chlorine number in molecule, and Chu and Hong's [1] database of PCB retention indices on DB-XLB capillary column. Similarly, the retention indices of 136 congeners were calculated using retention times (RTs) of PCBs on capillary column DB-1701 from Zhang's database [2]. The quality of proposed index system was proved by a comparison of predicted and experimental indices of selected congeners not involved in reference series. Afterwards the index systems were successfully used to identify several tens of individual PCBs both in river sediment and sewage sludge extracts, using only seven PCB standards.

Acknowledgement

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PERSISTENCE OF IMIDACLOPRID IN HORSE CHESTNUT WOOD AND LEAVES AFTER APPLICATION OF PREPARATIONS FOR LEAF MINER CONTROL

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Imidacloprid, 1-(6-chloro-3-pirydylmethyl)-N-nitroimidazolidin-2-ylideneamine, belongs to chloronicotinyl class of active ingredients. Imidacloprid acts as antagonist by binding to postsynaptic nicotinic receptors in the insect central nervous system. It has a broad spectrum of activities having contact and stomach action. Imidacloprid is readily taken up by plants and further distributed acropetally with good root systemic action. Since 1993 imidacloprid is used to control of leaf miner in horse chestnut tree by trunk application.

During 2003-2005 experiments were carried out in Łazienki, Warsaw and in Nieborów using imidacloprid preparations in form of gel (trunk application) and in solution (watering) to leaf miner control. Samples of wood and leaves from treated trees were collected and analysed in 2005. The residues of imidacloprid were quantified by reverse phase HPLC with diode array detection system after extraction of samples with acetonitrile-dichloromethane and purification of the extracts by column chromatography with silica gel. Two years after application imidacloprid was still found in wood on the level up to 0.9 mg/kg. The concentration of imidacloprid in leaves after 3-6 months after application was usually lower than LOD of analytical method, 0.02 mg/kg.

DETERMINATION OF POLYHYDRIC ALCOHOLS AND THEIRS CHLORODERIVATIVES BY MEANS OF GAS CHROMATOGRAPHIC METHODS

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Chloroderivatives of glycerol such as dichloropropanols and chloropropanodiols finding a wide application is many branches of the chemical industry e.g. for production of glycidol and epichlorohydrin. Dichloropropanols and chloropropanodiols are obtained in the hydrochlorination process of glycerol. For these synthesis can be used a waste glycerol obtained from the manufacture of biodisel and a waste hydrogen chloride obtained in the process of hydrocarbons chlorination.

The application of GC technique enables the determination of substrate conversion and the evaluation of yield of the final products.

The determinations of the products were carried out using the gas chromatograph Carlo Erba 8000 equipped with a flame ionization detector on a DB-WAX capillary column (length 30 m, I.D. 0.25 mm, film thickness 0.5 μm). The detector temperature was 250°C, the split-splitless sample injector temperature was 200°C, flow rate of carrier gas (helium) 2 $ml \cdot min^{-1}$, split ratio 1:10, sample injection volume 0.1 μl. The column temperature was programmed: initial temperature 100°C (hold for 5 min), temperature ramp rate $10°C \cdot min^{-1}$, final temperature 240°C (hold for 6 min).

The quantitative analyses were performed using the method of internal standard with the application of multilevel calibration. A statistical evaluation of elaborated method was performed. An accuracy and precision of this method was determined.

The structure of products were confirmed by GC/MS method using the gas chromatograph Trace 2000 Thermo Finnigan coupled to a quadrupole mass detector MS Voyager operating in EI mode at 70 eV (TIC mode, range 30–650 da) on a DB-1 capillary column (length 30 m, I.D. 0.32 mm, film thickness 1 μm). All compounds were identified on the basis of their mass spectra, comparison of retention times to these of standard compounds and interpretation of MS fragmentation patterns.

DETERMINATION OF *O*-ALKYL DERIVATIVES OF POLYCYCLIC ALCOHOLS BY RP-HPLC METHOD

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The allyl ethers of polycyclic alcohols based on the rosin structure are a group of compounds possessing very special properties. The derivatives are produced by *O*-alkylation on the phase transfer catalysis routes.

The determinations of allyl ethers of polycyclic alcohols were carried out using a Hewlett Packard HP 1090M liquid chromatograph. Chromatographic separation were performed on the analytical column (125 mm x 4 mm) filled with Purospher RP 18e of 5 μm particle size. Temperature of the column oven was set at 25°C. The mobile phase was a binary mixture of methanol and water (80:20 v/v) using linear gradient elution from 80 to 100% of methanol in the time of 30 min. The flow rate of the mobile phase was 1 mL·min^{-1} and the injection volumes were 10 μL of the samples. The range of wavelengths scanned was 190-600 nm. Analytes were monitored by UV-DAD detector at 230 nm to 18 min and 205 nm from 18 to 40 min. All compounds were identified using the UV spectral data. The structure of products was confirmed by GC/MS method.

EVALUATION OF SELECTED HERBICIDES RESIDUES IN SEDIMENTS BY RP-HPLC METHOD

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Atrazine and metamitron as s-triazine herbicides are widely used for weed control. Although there have been a few studies on the sorption, degradation rate theses herbicides on soils, there is little information on the sorption of these herbicides on sediments. The objective of our investigations was to develop and apply an analytical method for determination of atrazine and metamitron on a lake sediment.

Soxhlet extraction with acetone was used for extraction of herbicides residues from soil, followed used liquid-liquid extraction with dichloromethane and Florisil column chromato-graphy clean-up. This procedure was compared with thoroughly mixing of soil samples by shaker.

The determinations of the herbicides were carried out using a Hewlett Packard HP 1090M liquid chromatograph equipped with Rheodyne injector (injection loop of 10 μl) and UV-DAD detector on a Nucleosil 100 C18 column (150 x 4.6mm I.D., 5μm) under isocratic conditions. A mixture acetonitryle-water (60:40 v/v) at flow rate 0.5 $mL \cdot min^{-1}$ was used finally as a mobile phase. Temperature of the column oven was set at 25°C. The range of wavelengths scanned was 190-600 nm. Analytes were monitored by UV-DAD detector at 206 nm for metamitron and 222 nm for atrazine. Under these conditions the retention times of metamitron and atrazine were 7.87 min and 19.48 min, respectively. In the DAD system, purity analysis made by comparing the UV spectral data of the sample peaks with those obtained for the herbicides standards. The calibration solutions were prepared directly before performing the determination. The method has been validated for accuracy, precision, selectivity and linearity.

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APPLICATION OF ION CHROMATOGRAPHY FOR THE DETERMINATION OF INORGANIC ANIONS AND CATIONS IN DRINKING WATER ON INDIVIDUAL PURIFYING STAGES

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Water, as one of the most important environmental components, determine basic of life all organism. Water intended for drinking should be harmless to health, colourless, transparent and tasty. It has to meet a number of strict criteria, specified in appropriate directives. Drinking water must not contain an excessive amount of ferrous and manganese salts, pathogenic bacteria or suspended solids. However, the adequate proportion of macro- and microelements essential for human body, e.g. calcium, magnesium, sodium, potassium, copper and zinc is beneficial for human organism.

Górnośląskie Przedsiębiorstwo Wodociągów (The Upper Silesia Water Board) in Katowice is the main deliverer of drinking water for over 3 million residents of Silesia Province. The Silesian Waterworks administer eleven exploiting water purifying stations, which drawing water from surface intakes (7 stations - Goczałkowice, Strumień, Czaniec, Dziećkowice, Maczki, Będzin, Kozłowa Góra), and from drilled wells (4 stations – Bibiela, Miedary, Zawada, Łazy). Potential production is over 1,5 mln m^3/day. Depending on the kind of intake, water is characterized by different physico-chemical composition, affecting the appropriate choice of purification method.

Water samples from the Goczałkowice Waterwork were analyzed. This station drawing water from the largest Goczałkowice reservoir and from Czaniec reservoir. The main inorganic components of drinking water – cations (Na^+, K^+, Li^+, NH_4^+, Ca^{2+}, Mg^{2+}) and anions (F^-, Cl^-, Br^-, NO_3^-, SO_4^{2-}, PO_4^{3-}) were determination by ion chromatography method. All determinations were done according to the current standards: PN-ISO 10304-1 i 14991.

The research carried out allowed to compare of contents of inorganic compounds on individual purifying stages, to asses the quality of drinking water directed to the main water line and next to consuments and to check if it meets the conditions included in appropriate directives.

GC/MS DETERMINATION OF BIOLOGICALLY ACTIVE SUBSTANCE TRACES IN WATER PURIFICATION SEDIMENTS

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Apart from mineral compounds dissolved in them, surface waters include numerous organic substances. These can be natural products of decomposition of plant and animal organisms, synthetic substances from waste waters, and run-off from cultivated farmland and roads.

The present research focused on the sediments produced in the water purification process, following an observation that many organic compounds were adsorbed on their surface. We collected sediments from the coagulation of suspended matter, sediments from filters; we also analyzed extracts from active carbon filters used at the Gocza³kowice Water Utility Plant. These analyses were correlated with analyses of water samples collected at the subsequent stages of the production of potable water.

We determined the presence of neutral and acidic drugs and pesticides, but also fatty acids, phthalates and other plasticisers. Also detected were a number of substances not previously identified, such as Parsol, employed as a UV filter in creams, but since recently also a popular shampoo component.

Dried sediments (1-3g) were mixed with florisil and extracted three times with acidified methanol in ASE 200 at the temperature of 120°C under the pressure of 120 atm.

The extracts were evaporated to 0.1-1mL, derivatizated, and analyzed by GC/MS.

All analyses were carried out with a GC/MS GC Trace gas chromatograph coupled to an MS – MS Trace Finnigan detector on an Equity 5 (Supelco) capillary column, 30m x 0.25 mm x 0.25µm. The quantitative analysis was carried out by means of the SIM technique.

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EFFECTIVENESS OF REMOVING TRACE AMOUNTS OF DRUGS AND PESTICIDES IN THE WATER PURIFICATION PROCESS AT THE GOCZAŁKOWICE WATER UTILITY PLANT

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Surface waters constitute the main source of potable water in the Upper Silesia Region. In previous years, numerous drugs and pesticides were found in river waters. The following acidic and neutral drugs were determined: ibuprofen, ketoprofen, diclofenac, carbamazepine and pesticides: 2,4 – 2D, Metolachlor, Atrazine, Simazine, Terbutryne and Terbutylazine.

The aim of the present research was the monitoring of the water purification process at the Goczałkowice Water Utility Plant, which takes water from the Vistula and Soła rivers.

Water samples were drawn at eight sampling points along the water purification installation, starting with raw water, water after coagulation, filtration, ozonization, purification on active carbon filters, and finally pure water from potable water reservoirs.

It was found that initial ozonization and coagulation processes alone achieve a significant decrease in the concentrations of the compounds determined. After final ozonization, drugs and pesticides are no longer detectable.

Water samples were concentrated by the solid phase extraction (SPE) technique with the aid of BAKERBOND spe^{TM} C-18 POLAR PLUS columns conditioned with hexane, acetone, methanol and water. The extraction was conducted with water of pH = 4. The eluates were evaporated to a volume of 0.1mL. All analyses were carried out with a GC/MS GC Trace gas chromatograph coupled to an MS – MS Trace Finnigan detector (ThermoQuest) on an Equity 5 capillary column, 30m x 0.25 mm x 0.25μm. The quantitative analysis was carried out by means of the SIM technique.

The procedure employed allowed the determination of the analysed pesticides at the ng/l level. Highest concentrations were found for Metolachlor, Terbutylazine, and 2,4 – D.

Literature:

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PROBLEMS IN THE DETERMINATION OF PCB's IN SOIL AND SEDIMENT SAMPLES BY GC-ECD

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Polychlorinated biphenyls had been used until the 1960's as dielectrics in transformers and capacitors. Despite an early ban on their use, PCB's still present a significant environmental hazard due to their high chemical stability.

The determination of PCB's in typical environmental samples such as water, oils, and soil is documented in standarized procedures, e.g. PN-EN ISO 6468, PN-C-04579-1, and usually does not present analytical problems.

The determination of PCB's is carried out with relation to selected PCB congeners: 28, 52, 101, 118, 138, 153, 180; or commercial mixtures: Aroclor 1242, 1254, and 1260.

Examined samples of soils and sediments were analyzed by the Clarus 500 gas chromatograph with ECD detector and Elite-1 capillary column (30m x 0.32mm x 0.3μm). Temperature program: isothermal 80°C (1min), 80° - 280°C (8°/min), 280°C (50min).

Samples from waste stockyards as well as some bottom sediments revealed significant levels of lindane, which under standard analytical conditions has identical retention time to PCB-28, and can thus confound the analysis.

Similar confounding effects are introduced by the presence in the sample of other chlorinated pesticides, such as methoxychlor, polychlorinated phenols, especially PCP (pentachlorphenol), and polychlorinated terphenyls (PCT).

As PCB's have never been used as individual congeners, samples which chromatographic profiles significantly departing from the chromatographic profiles of Aroclors are always subjected to verification by GC/MS or analyzed under different chromatographic conditions.

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PLENARY SESSION

LIQUID CHROMATOGRAPHIC DETERMINATION OF GLYOXAL AND METHYLGLYOXAL FROM SERUM OF DIABETIC PATIENTS USING MESO- STILBENEDIAMINE AS DERIVATIZING REAGENT

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Meso-stilbenediamine has been used as derivatizing reagent for liquid chromatographic (LC) determination of glyoxal (Go), methylglyoxal (MGo) and dimethylglyoxal (DMGo) at pH 3. Liquid chromatographic elution and separation was carried out from the column Kromasil 100 C-18, 5µm (15 x 0.46mm id) with methanol : water : acetonitrile (59 : 40 : 1 v/v/v) with a flow rate of 1ml/min. and UV detection at 254 nm. The linear calibrations curves were obtained for Go, MGo and DMGo within 0.97-4.86µg/ml, 1.52-7.6µg/ml and 1.41-7.08µg/ml with detection limits of 48ng/ml, 76ng/ml and 70.8ng/ml respectively. The method was applied for the determination of Go and MGo from serum of patients suffering from diabetics and ketosis. The amount of Go and MGo found were 0.150-0.260µg/ml and 0.160-0.270µg/ml with coefficient of variation 2.6-4.7 %and 2.5-4.6 % respectively. The results obtained were compared with normal subjects with Go and MGo contents of 0.025-0.065µg/ml and 0.030-0.070µg/ml with C.V 1.5-4.9 % and 1.6-4.8 % in the serum.

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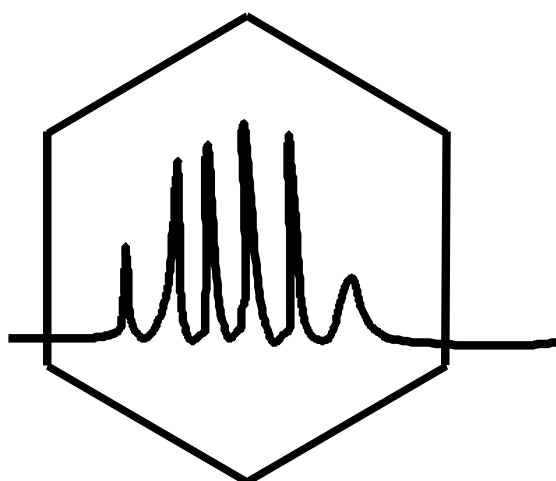

THE JUBILEE XXXth SYMPOSIUM

'CHROMATOGRAPHIC METHODS OF INVESTIGATING THE ORGANIC COMPOUNDS'

JUNE 12th – 14th, 2006
KATOWICE – SZCZYRK

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USE OF HPLC IN NONDENATURING CONDITIONS FOR THE STUDIES OF DNA QUADRUPLEX FORMATION AND DNA-LIGAND INTERACTIONS

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Introduction

Guanine-rich oligonucleotides are able to form a variety of multi-stranded non-canonical structures recognized as guanine-tetraplexes, G-quadruplexes, or G4 DNA [1-3]. Gellert et al. [1] first proposed the tetraplex structure for guanosine gels in 1962. Further interest in G-quadruplex structures was stimulated when several biological important guanine-rich genomic regions were discovered including telomeres, immunoglobulin switch region and some oncogene promoters [4-8]. Because of unusual molecular structure and potentially extensive cellular functions, G-quadruplexes represent a very attractive target for drug design [9-13].

The G-quadruplexes exhibit four-stranded structures containing one or more nucleic acid strands, in parallel or antiparallel orientations, with a central channel able to accommodate metal cations [8]. Four guanines on a plane, interacting via Hoogsteen bonding, form a G-quartet as shown in Fig. 1A. Typically, two, three or four G-quartets are stacked within a quadruplex and held together by π-π nonbonded attractive interactions. Guanine quadruplexes exhibit polymorphism. The overall topologies available to G4 structures depend on many factors including strand stoichiometry and polarity, glycosidic torsion angle, connecting loops, and coordination of cations [8]. Last parameter, the selective coordination of selected metal cations in a central channel formed by stacking of guanine tetrads, plays very important role in quadruplex stability and topology [2,14].

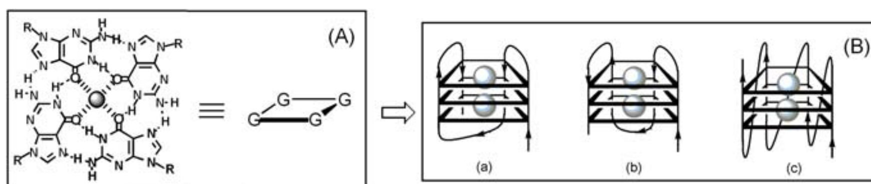

Figure 1. (A) Structure of G-quartet showing hydrogen bonds between four guanines and the interaction with a cation (circle). (B) Schematic representations of G-quadruplexes: an antiparallel “basket-type” form (a), an antiparallel “chair-type” form with all lateral loops (b), and a parallel quadruplex with all loops positioned alongside the grooves (c).

For example, the sodium quadruplex formed by the human telomeric DNA sequence, d[AGGG(TTAGGG)$_3$], possesses antiparallel strand orientation with one diagonal and two lateral TTA loops at the G-quartet ends. This resulted in the basket-type structure (a) in Fig. 1B [15]. On the contrary, the intramolecular potassium complex with human telomeric sequence exhibits a variety of G-quadruplex structures ranging from antiparallel basket- or chair-type

structures ((a) and (b) in Fig. 1B) to a controversial parallel conformation (c) called also a propeller-type structure. The latter shows a strikingly distinct arrangement, with all three TTA loops run on the outside of the guanine tetrad core (alongside the grooves) [16,17]. Recently, Chaires et al. presented results obtained with a variety of experimental and calculation methods, which are inconsistent with the existence of propeller-shaped structure in solution [18]. Thus, the formation in solution of parallel G-quadruplexes by human telomeric sequence remains controversial.

Several techniques are employed to explore formation and properties of G-quadruplexes including gel electrophoresis, X-ray diffraction, NMR spectroscopy, circular dichroism (CD), mass spectrometry, and UV-Vis spectroscopy. Recently, we have reported preliminary results on the application of HPLC method, which has potential to be useful for separation of different structures of G4 DNA and may clarify the controversy on the existence of a propeller-type potassium quadruplex in aqueous solution [19,20].

In this report we descried the effect of metal cations on quadruplex formation, as well as an attempt to monitor the interactions of DNA ligand, ethidium bromide (EtBr), with G4 DNA and double-stranded DNA (dsDNA).

Experimental

The 21-mer quadruplex-forming DNA oligonucleotide with human telomeric sequence $dG_3(T_2AG_3)_3$ (G4 DNA) and its complementary strand $dC_3(TA_2C_3)_3$ (C4) were synthesized and HPLC-purified by Genosys, Sigma. Purity of oligonucleotides was examined by MALDI TOF mass spectrometry. In experiments with double-stranded DNA, the comple-mentary DNA strands were mixed in desired amounts and the total species concentrations were confirmed by molar absorptivity of the dsDNA. All experiments were performed in a buffer solution consisting of 20 mM TRIS at pH 8.7 unless otherwise indicated. Other reagents were of analytical-reagent grade and were used as received. A Milli-Q filtered water (Millipore, Bedford, MA) was used throughout.

HPLC separations were carried out on a Hitachi LaChrom HPLC system composed of a L-7350 column oven, a L-7100 pump, L-7455 diode array detector, and D-7000 HPLC interface. Separations were performed at 27.5^{o}C on a TSKgel DEAE-NPR column (4.6 mm i.d. x 3.5cm; d_p = 2.5μm) (Tosoh Bioscience, Tokyo, Japan). Separations were carried out using gradient elution with a flow rate of 1 ml min^{-1}. The mobile phase contained 20 mM Tris-HCl buffer pH 8.7 and desired metal chloride concentration. Gradient elution was accomplished with 1M LiCl (0.1 to 0.7 M LiCl in 15 min).

Results and Discussion

Separation of guanine quadruplexes is routinely achieved with the use of gel electro-phoresis. To our best knowledge, there are no literature reports on the application of chromato-graphic methods for this purpose except for our reports [20,21]. Such approach seems to be very attractive if one considers rapidly developing hyphenated techniques, which may allow precise spectral characterization and identification of particular quadruplex forms.

To achieve separation of quadruplex forms, the nondenaturing conditions of HPLC experiment were maintained. We have chosen the high performance ion-exchange chromato-

graphy (IE HPLC), which is widely used for separation of double-stranded DNA fragments. Short columns (2-5 cm) with stationary phases containing micropellicular particles (1-3 μm) are useful for separation of dsDNA analytes [21]. A lithium chloride gradient was used to elute quadruplexes since Li^+ is believed to be a poor quadruplex stabilizer [2]. Four metal cations were tested for the quadruplex-forming abilities: K^+, Na^+, Li^+ and Mg^{2+}.

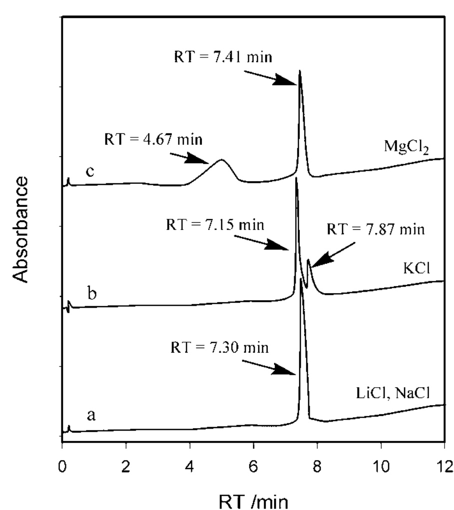

Figure 2. (A) Chromatograms of G4 quadruplexes eluted with LiCl gradient (0.1 to 0.8 M LiCl in 15 min). Mobile phase contained: (a) 0.1 M NaCl or LiCl; (b) 0.05 M KCl; (c) 0.05 M $MgCl_2$. Conditions: column TSKgel DEAE-NPR (4.6 mm i.d. x 3.5 cm; d_p = 2.5 μm), flow rate 1 ml/min, 20 mM Tris buffer pH = 8.7; λ = 260 nm.

Figure 2 shows the effect of metal cations on the quadruplex separation. In the presence of LiCl and NaCl (a in Fig. 2), only single peak is eluted with approximately the same retention time for both cations (RT = 7.3 min). The result for NaCl is consistent with our earlier observations [20,21] and literature reports on a single form of sodium quadruplex (form a in Fig. 1B). More complex chromatograms were obtained with K^+ ((b) in Fig. 2), and Mg^{2+} ((c) in Fig. 2), where two peaks can be recognized. One peak is eluted around 7.3 min and can be assigned to the antiparallel form, similarly as for Na quadruplex. The second peak for KCl separation (RT = 7.87 min) suggests existence of a parallel structure ((c) in Fig. 1B). A broad second peak eluted in the presence of $MgCl_2$ (RT = 4.67 min) may represent more complex oligonucleotide mixture, including random coil arrangement.

We have tested also a suitability of IE HPLC for the monitoring of dye-DNA interactions. Well-known DNA intercalator, Ethidium bromide (EtBr), was chosen as a pilot ligand and sodium complex of G4 DNA as a model quadruplex. Interaction of dye with dsDNA, obtained by titration of G4 strand with the complementary oligonucleotide C4, was also examined. Results of these experiments are shown in Fig. 3A and 3B. No interaction of EtBr with sodium quadruplex was observed as indicated from a flat line, when monitoring

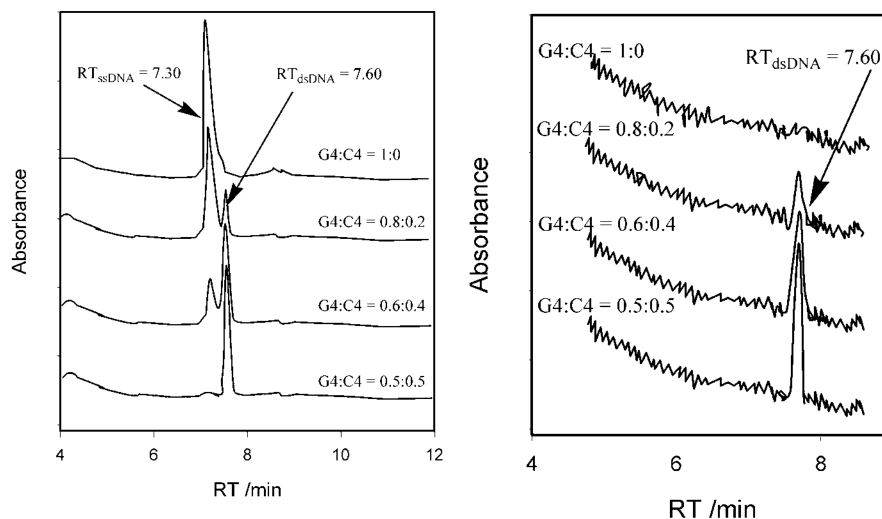

Figure 3. HPLC separation of G4 DNA ($dG_3(T_2AG_3)_3$) titrated with the complementary strand C4 ($dC_3(TA_2C_3)_3$): monitored at 260 nm (A) and 520 nm (B). Conditions: column TSKgel DEAE-NPR (4.6 mm i.d. x 3.5 cm; d_p = 2.5 µm), flow rate 1 ml/min, 20 mM Tris buffer pH = 8.7, 0.1 M NaCl, 5 µM EtBr, chloride gradient: from 0.1 M to 0.8 M LiCl in 15 min.

wavelength was set at the EtBr band of 520 nm (Fig. 3B, G4:C4 = 1:0). On the contrary, titration with C4 strand revealed formation of a second peak corresponding to dsDNA (RT = 7.60 min), which was reproduced in chromatograms recorded at 520 nm (Fig. 3A and 3B).

These results clearly shoved, that IE HPLC could be successfully used for investigation of ligand-DNA interactions at least with dsDNA. Quadruplex – ligand interactions need further studies with the participation of stronger binding ligands.

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APPLICATION OF GAS CHROMATOGRAPHIC METHOD IN HELIUM CONCENTRATION MEASUREMENTS IN GROUNDWATER FOR HYDROLOGICAL PURPOSES

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Key words: gas chromatography, hydrology, helium, groundwater dating.

Abstract

A chromatographic measurement system of helium concentration in groundwater is presented. Water samples are taken from groundwater with a precise procedure without contamination with air in a special glass vessels of volume equal to 1180 cm^3. Helium is extracted from water samples using the head-space method. After enrichment by cryotrap method helium is analysed in the gas chromatograph equipped with the TCD detector with detection limit of about $1.6 \cdot 10^{-12}$ gHe/cm^3_{air}.

Introduction

Helium concentration in groundwater is a fine indicator in water dating in a range from a hundred to tens of thousands of years and ^4He is also used for dating young waters[1,7]. One of the advantages of the helium method is its considerably wider range of dating in comparison with, for example, the ^{14}C method. In some cases the former method ensures even more reliable results than the ^{14}C method[2].
Helium concentration in the atmosphere is about 5.24 ppm. Knowing helium solubility in water, one can calculate the concentration of helium in surface water using the Henry's law[3]. Surface water in equilibrium with the atmosphere and at the temperature of 20°C contains about $8.6 \cdot 10^{-12}$ gHe/cm^3_{H2O}. Helium concentration can be increased in groundwater as a result of excessive air getting into the water or as a diffusion and accumulation in water helium which is non-atmospheric. This non-atmospheric excessive helium can originate from: (a) in situ production in rock material as a result of presence of radioactive isotopes: ^{238}U, ^{235}U i ^{232}Th; (b) diffusion release of helium accumulated in sandy grains; (c) outside flux[4]. The chromatographic method should allow the analysis of helium at the level of $8.6 \cdot 10^{-13}$ g/cm^3_{H2O}.
The age of groundwater can be calculated from equation (1)[1,4]:

$$Age(^4He) = C_{He} \cdot n \cdot h \cdot \rho_f / (J + n \cdot h \cdot \rho_f \Lambda \cdot P) \qquad (1)$$

where C_{He} is measured non-atmospheric excessive ^4He, n – total combined porosity, h – depth of water-bearing layer, J – netto Earth's flux of ^4He to water-bearing layer, ρ_f - water density, Λ - fraction of gas released from rock, P – in situ production of ^4He.

Young waters have the ^4He concentration very similar to the concentration in equilibrium with the atmosphere, for example most waters from Czatkowice sources and Ruszcza waters near Kraków [5].

For the concentration of uranium (C_U) and thorium (C_{Th}) given in ppm, the rate of in situ production of ^{4}He P (in cm^{3}STPg^{-1}year^{-1}) is given by equation (2) [1,4]:

$$P = (\rho_r/n)(1.19\cdot10^{-13}\cdot C_U + 0.288\cdot10^{-13}\cdot C_{Th}) \qquad (2)$$

where ρ_r – rock material density.

In literature one can find the value of the Earth's stream of ^{4}He inclusive the range of (0.2-8.2) 10^{-6} with the average value about $2.4\cdot10^{-6}$ cm^{3}STPcm^{-2}year^{-1} or J = $(1.0\pm0.4)\cdot10^{-6}$ cm^{3}STPcm^{-2}year^{-1}, but in some groundwaters the domination of in situ production was stated, what in this cases sugests well sealed ground and (or) carrying of helium which migrates from the depth of the earth by lower situated water system.

Applications of helium method known so far, consisted of ^{4}He concentration measurements using special mass spectrometers[6]. Such measurements unfortunately are very expensive and not available in Poland. That is why this problem is tried to be solved using much cheaper gas chromatographic method. For waters in equilibrium with the atmosphere in temperature of 10°C, the rate of ^{3}He/^{4}He amounts to $140\cdot10^{-8}$ and is often signified by R_A. Helium originated only from the Earth's crust should have R_c/R_A. = 0.01, and one that originates only from the Earth's mantle R_m/R_A = 8. This datasets show that $(^3He/^4He)_c = 140\cdot10^{-10}$ and $(^3He/^4He)_m$ = $1,1\cdot10^{-5}$, so the highest concentration of ^{3}He = $1.12\cdot10^{-5}\cdot{}^4$He. Measurements of ^{4}He for dating waters can be substituted for the measurements of total helium concentration, because concentration of ^{3}He is in this case considerably low.

Experimental

A scheme of the measurement system is presented in Figure 1.

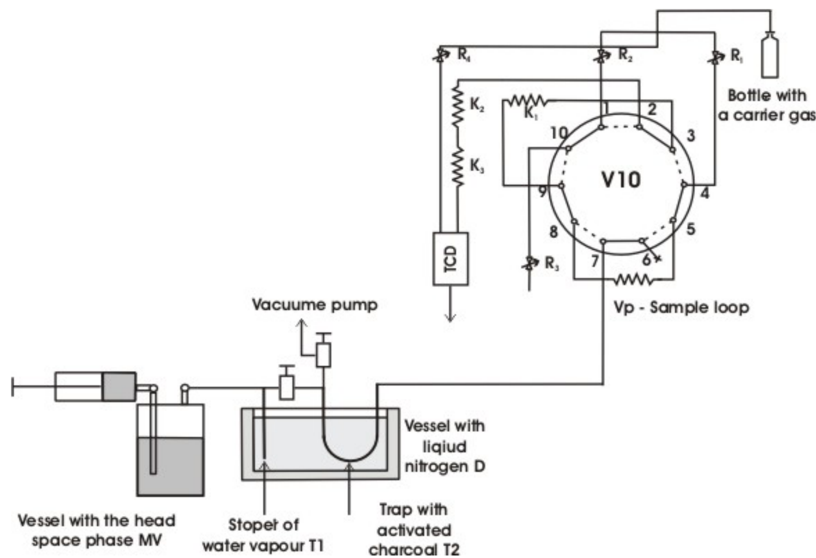

Fig. 1. Scheme of the chromatographic system for measurements of helium concentration in groundwater (description in the text).

Measurement system consist of Shimadzu gas chromatograph equipped with a TCD detector of 2µl volume; 10 port valve V10; three chromatographic columns K1(1.5 m), K2(7 m) and K3 (2m); sample loop V_p; system of helium enrichment and the vacuum pump [8,9,10,11, 12]. Helium is extracted from water samples using the head-space method described by Śliwka and Lasa[13]. The gas sample obtained using this method passes through a system of two (vacuumed earlier) traps immersed in liquid nitrogen, D. In the first trap T1, the water vapor from the sample is stopped. In the second trap T2 filled with activated charcoal, the oxygen and nitrogen are adsorbed. The helium not adsorbed fills the volume from the trap T2 to the end of the sample loop also vacuumed earlier. After changing the position of V10 the sample is dosed to the first column K1(filled with molecular sieve 5A). When helium gets to the second column K2 (filled with molecular sieve 5A) the position of V10 is changing back and the compounds which remained in K1 are removed from the system. The columns work in the "back flush" mode. For better separation helium and neon pass through the third column K3 (filled with a mixture of molecular sieve 5A and activated charcoal(50%/50%)), and gets to the TCD detector. The signal from the detector is registered in a computer equipped with the appropriate software.

Results

The difficulties with measurements of helium concentration in water derive from: (a) lack of proper detector for He detection and (b) difficulty with separation helium from neon in room temperature. Peaks of helium and neon appear together, when columns are in room temperature. There are two ways to separate them and these two ways where applied. Firstly, there were used short (K1-1.5m., K2-3m., K3-2m) columns in temperature: – 80°C, and secondly, columns were longer (K1-1.5m., K2-7m., K3-2m) in room temperature. Additionally, application of a third column K3 packed with a mixture of molecular sieve 5A and activated charcoal, gave advisable effects.

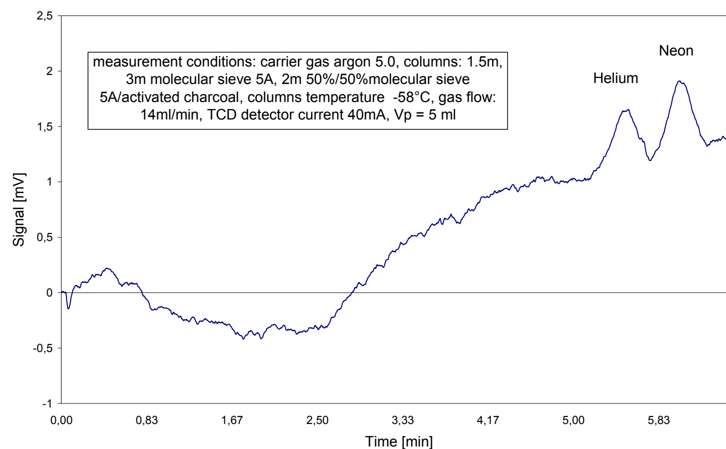

Fig. 2 Result of the direct measurement of helium concentration in the atmosphere using the gas chromatographic method.

The detection limit for the TCD detector obtained in this system is $1.6 \cdot 10^{-12}$ g/cm$^3_{air}$. That is why a system of enrichment in liquid nitrogen had to be used.

The results from the analyses of helium concentration in groundwater and in the atmosphere, obtained through the chromatographic method described above are shown in Figures 2,3 and 4. Result from measurement of helium concentration in the atmosphere without enrichment is shown in Figure 2. In Figure 3 and 4 results from measurements of helium concentration in the atmosphere and the analysis of helium concentration in water with enrichment in a trap T2, are presented respectively.

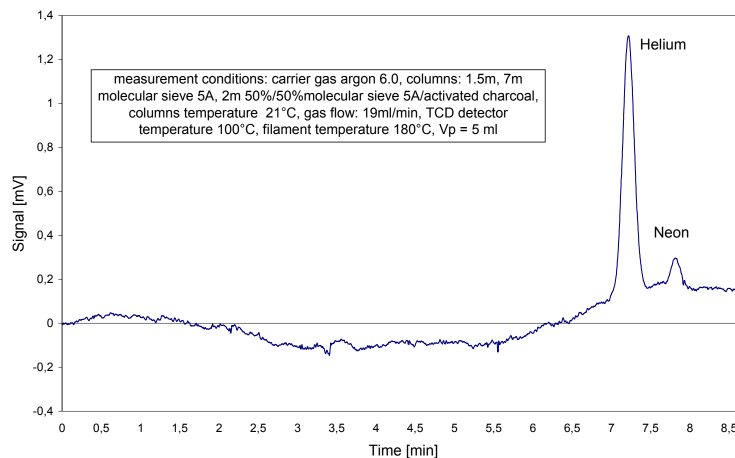

Fig. 3 Result of the measurement of helium concentration in the atmosphere using the gas chromatographic method with enrichment in the trap T2.

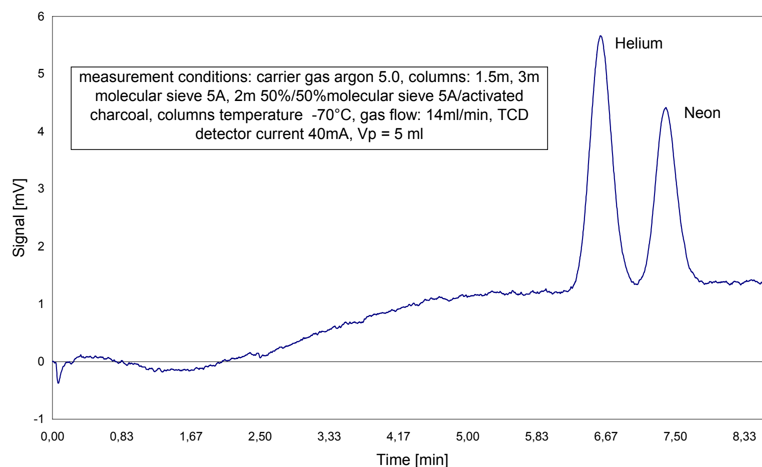

Fig. 4 Result of the measurement of helium concentration in water using the head-space method with enrichment in the trap T2.

Conclusions

The developed chromatographic system described here, can be used for measurements of helium concentration in groundwater.

The obtained level of helium detection for water samples of volume equal to 1180 cm^3, where helium was extracted to 100 cm^3 head-space gas phase, amounts to $8 \cdot 10^{-13}$ g/cm^3_{H2O}.

Acknowledgments

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FREE RADICALS, TOTAL ANTIOXIDANT POTENTIAL AND THEIR ANALYSIS USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Introduction

Although oxygen is necessary to live it is also toxic for living organisms [1, 2]. A big part of oxygen is used as electron acceptor. Some enzymes catalyze oxidation reactions of transform electrons from substrates on oxygen molecule. During its reduction the so called ROS - reactive oxygen species are created. Among them one can enumerate free radicals and also singled oxygen and hydrogen peroxide. Free radicals are atoms, ions, molecules or their fragments having unpaired electrons, giving them paramagnetic properties. They are present in every living cell [3, 4]. Their concentration in animal tissue (about 10^{15}/g) change widely after interactions with some chemical compounds, radiation, sickness, stress (using much more oxygen) and aging. In the living organisms free radicals are generated in physiological as well as pathological conditions mainly on the inner surface of the mitochondrial membrane (Table 1). In physiological they do not accumulate in tissue because they are scavenged by the local antyoxydative mechanisms. In the pathological conditions they can cause degeneration and even cell deaths. Free radicals interact with all cell components. The biggest changes they cause in lipids, proteins and DNA (Table 2). From the cell organs the most exposed on the free radicals attack are mitochondria. They damage may cause even cell death. Half spin particles are described by the Fermi-Dirac statistic. According to the probability function the electron (fermion) appearance increase with decrease of energy and never over cross 1. It means that in a given energetic (described by energy, momentum and spin) state may be only one electron. The electron disposition in the oxygen molecule and its reduction products is presented in Fig. 1. In the basic state the oxygen is a triplet biradical, what explains its relatively high reactivity and in the same time small as for radical.

Table 1. Generation of the Free Radicals in Living Organisms

1. Exposition on high and low oxygen pressure and after ischemic reperfusion
2. Interaction of chemical compounds (xenobiotyks, pesticides (paraquat), tetrachlorocarbon, PCH, anthracyclic antibiotics, ozone, nitric oxides, nicotine)
3. Energetic changes (α, β, γ, UV, visible, IR, X radiation, electrical discharges)
4. Metabolic processes in microsomes, mitochondria, during phagocytose, photosyntese, cytochrome reactions, peroxydation of PUFA
5. Disorders of metabolic processes by avitaminose (A, E and C), aging, sickness (Alzheimer disease, tumor, rheumatism, diabetes, alcoholism, after encephalopathic blood deficiency) vitamin

Singled oxygen

Fig. 1 shows the products from the reduction of oxygen. In the basic state oxygen is a triplet biradical and in the excited state oxygen appears as a singlet which is present in cytoplasm at a concentration of about 10^{-15} M (compared to about 10^{-5} M for the triplet form). Two singlet oxygen states are known: delta ($^1\Delta gO_2$) and sigma ($^1\Sigma g^+O_2$). They are created during oxidation of superoxide anionradical with iron (Haber-Weissa reaction) and oxyalcohol, during phagocytose, nonenzymatic lipid oxydations, peroxide dismutation etc.

Table 2. Influence of Free Radicals on Basic Components of Animals Cells

1. Lipid peroxidation (changing of membrane "liquidity" and permeability, mitochondrium swelling)
2. Amino-acids and proteins (modification of aminoacids, peptides fragmentation cross linking)
3. Nucleic Acids (modification of heterocyclic bases and pentoze rests, tear of glikozyde and phosphodiester bonds)

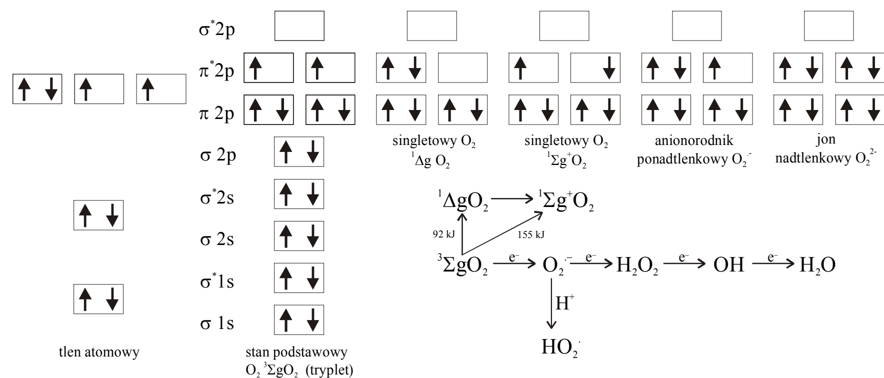


Fig. 1. Electrons distributions in oxygen and products of its reduction.
□ - molecular orbital, * - anti-bond orbital, ↑↓ - electron spines.

Superoxide anion-radical, O_2

The first products in the oxygen reduction scheme in Fig. 1 are the superoxide anion-radical ($O_2^{\bullet-}$) and its protonated form, the hydroperoxyl radical ($HO_2^{\bullet}$, pK_a = 4.88). The concentration of these species in the biological cell is about 10^{-11} M and is kept essentially constant by superoxide dismutase (SOD) [4]. In 1954 Gershman, Gilbert and Fridovich suggested that it is responsible for oxygen toxicity. However it turned out that although it is highly reactive, it is non-reactive in aprotic environment and for amino acids and lipids.

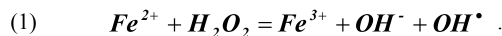
Hydroperoxyl radical, $HO_2^{\cdot}$

The hydroperoxyl radical is a much stronger oxidant than superoxide anion-radical and was initially thought to be responsible for the toxic effects of oxygen and cell membrane damage [5]. $HO_2^{\cdot}$ has a relatively long lifetime and so can diffuse to neighbouring structures. At the physiological pH of 7.4 about 1% of superoxide exists in its protonated form. A quantitative method for hydroperoxide is based [6] on the oxidation of glutathione (GSH) by hydroperoxide and formation of oxidized glutathione (GSSG). This reaction is catalyzed by glutathione peroxidase. GSH and GSSG were separated on reversed phase column at pH 3 and their concentrations were estimated using electrochemical detection at a glassy carbon electrode. The method was applied to the analysis of picomolar levels of many hydroperoxides in human plasma.

The next reaction product in Fig. 1 is hydrogen peroxide, which can be determined by a range of methods: for example, a detection limit of 6 ng can be obtained with fluorescence detection.

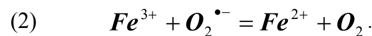
Hydrogen peroxide, H_2O_2

Hydrogen peroxide is generated during dismutation reaction. It is also generated by L-aminoacid and glikolan oxidases, which carry 2 electrons. In all other cases reaction goes in two steps. In the presence of transition metal ions (iron, cooper) hydrogen peroxide dissociated with production of very reactive hydroxyl radical, according to described in 1884 by Fenton reaction:

(1) 

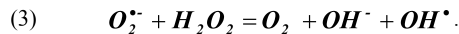
$$Fe^{2+} + H_2O_2 = Fe^{3+} + OH^- + OH^{\bullet}$$

Already oxidized metal can be reduced, it means that it plays role of catalyst:

(2) 

$$Fe^{3+} + O_2^{\bullet -} = Fe^{2+} + O_2$$

The both reactions give the Haber-Weiss one (1934 r.):

(3) 

$$O_2^{\bullet -} + H_2O_2 = O_2 + OH^- + OH^{\bullet}$$

Hydroxyl radical, $OH^{\cdot}$

Reduction of hydrogen peroxide gives the very reactive hydroxyl radical, $OH^{\cdot}$. This radical interacts with a number of organic compounds by addition, free radical substitution and electron transfer reactions. It does not penetrate the cell and interacts only with molecules that are in close proximity [7]. Its concentration in biological systems is of the order of 10^{-11} M. Because of their extreme reactivity, hydroxyl radicals are primarily analyzed using radical trapping agents followed by electron spin resonance or HPLC determinations. ESR is highly sensitive but is difficult to apply directly to biological systems. Free radicals may be investigated using this technique by transforming them into more stable species using the so called "spin trapping" method. When this method is used for biological analyses the radical trap agent should be selected extremely carefully to avoid toxicity problems. Examples of suitable compounds are phenylalanine (with which hydroxyl free radicals react to produce tyrosines [8]) or derivatives of aspirin (*o*-acetylsalicylic acid). In living organisms aspirin is hydrolyzed rapidly to salicylic acid, which under physiological conditions at pH = 7.4 reacts with hydroxyl radicals to give three main products: 2,3-dihydroxybenzoate (49%) and 2,5- dihydroxybenzoate

acid (40%) (collectively referred to as DHBA) and *o*-catechol (11%) (Fig. 2). These derivatives can be separated using reversed phase HPLC [9] (Fig. 3) and detected photometrically or more sensitively by electrochemical detection using a glassy carbon electrode at 0.8 V *vs* Ag/AgCl (detection limits of 1 pg for DHBA and 100 pg for salicylic acid) [10]. DHBA compounds are eluted near the column dead volume, together with other species such as catecholamines. For this reason, use of ion-pairing chromatography [11] is the preferred separation mechanism. Use of salicylic acid as the radical trap agent has some disadvantages [12], especially the determination of 2,5-dihydroxybenzoic acid due to endogenic production of this species by, for example cytochrome P-450, and the sensitivity of this system is reduced because there are two products of the reaction.

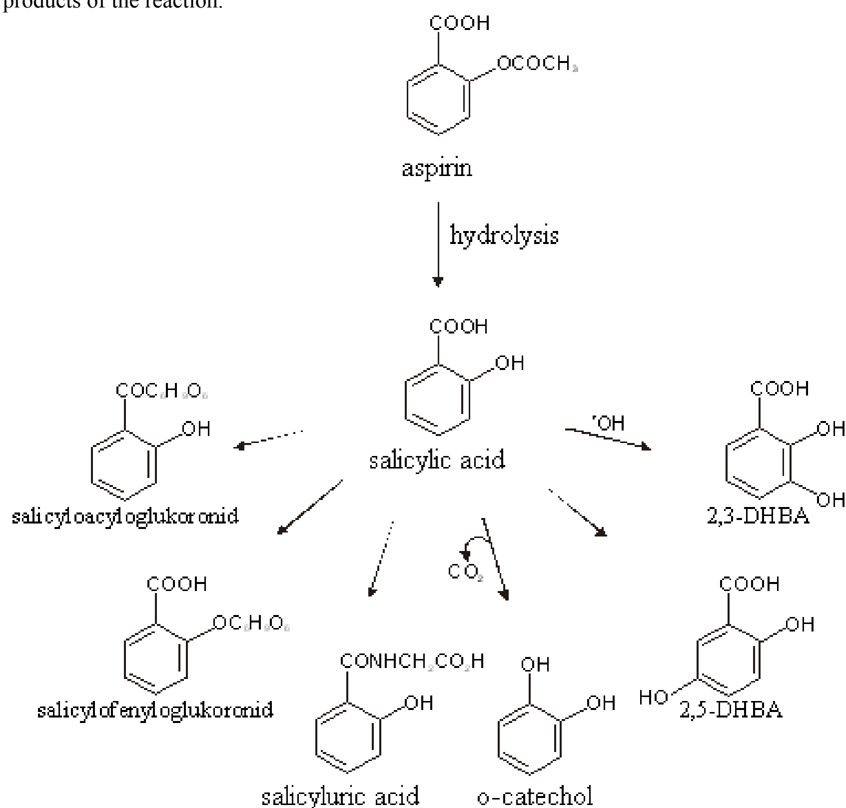


Fig.2. Metabolites and products of the reaction of hydroxyl radical with salicylic acid.

To avoid these problems Ste-Marie *et al.* have proposed the use of *p*-hydroxybenzoic acid as the radical trap agent [12] and this species has recently been applied using ion-exclusion chromatography to measure the product of reaction (3,4-dihydroxybenzoate) [11]. This method has a number of advantages, in that only one isomer is formed by hydroxylation, so the detection signal is not split between two compounds, and the separation is relatively simpler.

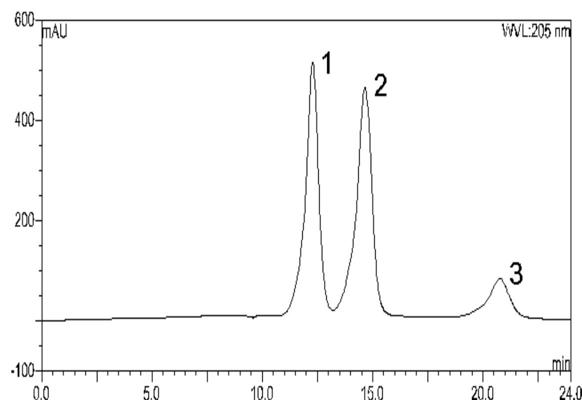
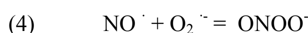


Fig. 3. HPLC chromatogram of 1 mM standards of acids (1) - 2,5-, (2) - 2,3-dihydroxybenzoi-cand (3) – salicylic acid. Chromatographic conditions: column - 250x4 mm I.D. Zorbax ODS 5µm (Knauer); mobile phase – acetate-citrate buffer pH 4,3, 1 mM KCl, 0,25 mM EDTA, 5% MeOH, 3mM TBAP; temp. - 20ºC; flow rate - 0,9 ml/min.; detector - UV-205 nm [11].

Nitric Oxide

Recently, there has been intense investigation of the free radical, nitric oxide [13, 14]. This species is a neurotransmitter (in the central as well as peripheral neuronal systems) and a free radical scavenger, but being a free radical it is also neurotoxic and generates other free radicals. The effects of NO are probably dependent on concentration, with high concentrations NO acting as free radicals, and low concentrations being protective. In the biological cell nitric oxide is generated during oxidation of arginine to cytruline (in the presence of NADPH as an electron donor) by nitric oxide synthase (NOS). In some tissues the only source of citrulline is NOS, thus the determination of citrulline provides the possibility to measure NOS activity and indirectly, the nitric oxide concentration. The standard method HPLC method for this determination is based on derivatisation with *o*-phthalaldehyde, followed by separation on a C18 stationary phase and fluorometric detection at the excitation/emission wavelengths of 338/425 nm. This method provides a detection limit of 0.1 pmole.

Nitric oxide is quickly degenerated due to interaction with oxygen, nitric dioxide and the superoxide anion-radical. It scavenges the superoxide anion-radical, generating peroxynitrite anion, as follows:



(4) $NO^{\cdot} + O_2^{\cdot -} = ONOO^-$

After protonation peroxynitrite decomposes into two radicals, hydroxide and nitric dioxide, and under physiological conditions it rearranges to nitrate in about 1 s. The neurotoxicity of nitric oxide arises from peroxynitrite and its decomposition products, which interact with lipids, DNA, and the protein tyrosine. In the latter case, the product (nitrotyrosine) may be determined relatively easily by reversed-phase HPLC with photometric (274 nm) or electrochemical detection, or by ion-exclusion chromatography together with dihydroxybenzoic acids. The determination of NO_2^- and NO_3^- also can be used as an indirect method for the measurement

of nitric oxide. These species are determined using ion-exchange and ion-pair chromatography, with conductometric, photometric (214 nm), and electrochemical detection (for nitrite) [2]. The high concentration of chloride (10^5 higher then nitrite) present in biological samples can be removed using a cation-exchange resin in the Ag^+ form, or by using chloride as a mobile phase. Good separation was also obtained using capillary electrophoresis.

Lipidic radicals

Free radicals interact with lipids producing other radicals and stable lipid peroxides [1], which usually are more reactive and toxic then the original substrate radicals. Non-metal enzymes (such as lipooxydase) also generate lipid radicals. These species do not contain strong chromophores and are electroinactive in the accessible potential range, but can be determined chromatographically after derivatisation. A sensitive analysis (at the ng level) uses derivatisation to *p*-hydroxyanilides, followed by separation on reversed-phase column and electrochemical detection at 0.7 V [26]. Hydroxyperoxides of phospholipids may be determined after derivatisation with 2,2'-azobis(2-amidynopropan)dihydrochloride, followed by separation on a propylamine stationary phase and chemiluminescence detection.

Degradation of cell membranes is observed during reaction of free radicals with polyunsaturated fatty acids, PUFA, to yield lipid hydroperoxides, aldehydes and ketones. These compounds are relatively stable and cytotoxic. They interact with the cell and membrane components, showing strong chemical affinity. Generated in blood (endothelium, fibroblasts, hearth muscle *etc*.) they are responsible for arteriosclerosis. Additionally they influence on the change of electric charge, hydrophobicity and "liquidity" of membranes decreasing their mobility. New electric charges on the membrane surface indicate on new carboxylic groups generated during peroxydation. It causes them more hydrophilic, what change the membrane structure (transport, receptors).

Estimation of oxygen radical damage in biological system is usually done by determination of malondialdehyde (MDA) [15]. The most frequently method used is the thiobarbituric acid (TBA) test. It has been favored assay because of its simple handling and sensitivity. This is true despite the fact that the TBA assay is intrinsically non-specific and is generally poor when applied to biological samples. Positive response is obtained with sugars, some amino and bile acids, alkenals, alkadienals *etc*. Certain improvement can be obtained using RP-HPLC separation followed by fluorometric or photometric detection at 535 nm [15]. In this case thiobarbituric influence both, the retention (separation) as well as detection conditions. This method still requires derivatization step. Additionally its results are questionable because identical adducts are yielded from different substances. Similar method is based on the derivatization with 2,4-dinitrophenylhydrazine [16] or diaminonaphthalene [17] followed by photometric detection at 310 nm. Simplification of this method (no derivatization is required and sample preparation is minimal) was shown by application reversed phase ion pair chromatography. Detection is accomplished by monitoring absorbance at 267 nm, enabled analysis of MDA together with two antioxidants, namely ascorbic and uric acids. Unfortunately, described method produce very broad and small peak of MDA.

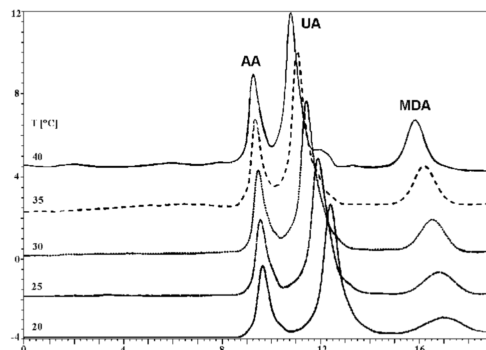


Fig. 4. IEC chromatograms of ascorbic acid (AA), uric acid (UA) and malonyldialdehyde (MDA) obtained at different column temperatures. Chromatographic conditions: column – 300 x 7.8 mm I.D. TSK-GEL SCH(H^+) (TosoHaas); mobile phase – 3 mN H_2SO_4, 3 mM TBABr, 5% ACN; detector UV-267 nm; temp. - 20°C ÷ 40°C.

Recently it was shown that ion exclusion chromatography can be applied to the analysis of malonyldialdehyde [15]. Two methods were compared. The first one was based on its derivatisation with thiobarbituric acid, the second one on direct its separation with ion interaction reagent added to the mobile phase. Better separation was obtained using second method although lower detection limit offered the first one. Peak broadening was strongly affected by column temperature, what is probably caused by kinetic effects (Fig. 4).

Total Antioxidant Potential

Because of the interaction between free radicals and antioxidants in pathology changes of antioxidants concentration are observed. Complementary the antioxidants concentrations can be measured [2, 18-20]. It turned out that estimation of total antioxidant potential (TAP) is frequently much more useful then separate analyzing all of them. Cooperation between different antioxidants gives sometime better protection than separate compounds. As an example of synergism we can mention glutathione, which regenerate ascorbate or ascorbic acid regenerating α-tocopherol. Both these magnitudes, *i.e.* oxidative stress and total antioxidant potential are correlated each other and even at times identified.

The ability to scavenge free radicals are defined differently and different names are used TAC – total antioxidant capacity, FRAP – ferric reducing ability of serum, TRAP – total peroxyl radical – trapping antioxidant parameter, TRAP – total redox antioxidant potential, ORAC – oxygen radical absorbance capacity, TEAC – Trolox-equivalent antioxidant capacity or TAR – total antioxidant reactivity [21 - 23]. In the paper it will be used most general name – Total Antioxidant Potential, TAP.

TAP has been introduced for evaluating, mainly, antioxidant capacity of complex biological samples, such as plasma. These tests adopt different free radicals generators (usually thermolabile diazocompounds generating peroxyl radical) and the oxidation of analyzed samples. Because of competition, sample delay interaction between detector and radical. Results are calculated from the delay time during which antioxidants are consumed. The basic requirement

of analyzed sample is that its oxidation rate constant is much higher then that of the compound used as a “detector”. The basic requirement of analyzed sample is that its oxidation rate constant is much higher then that of the compound used as a “detector”. Described in the literature method is based on the generation of peroxyl radicals and then fluorometric or chemiluminescence kinetic measurement of the product of its reaction with the so-called detector, i.e. easy to detect compound. As a detector usually it is used derivative of fluorescein, in the case of fluorometric measurements or luminol when chemiluminescence method is used [24, 25]. It was also described application of photometric assay to the estimation of antioxidant potential of cells [24]. In this case as a detector diacetate of dihydrodichlorofluorescein, DCFH-DA, was used. Esterase from cell converted it to dihydrodichlorofluorescein, DCFH. Finally, after reaction with peroxyl radical, dichlorofluorescein, DCF, was obtained. The last compound is characterized by strong adsorption at 520 nm.

HPLC measurements of the Total Antioxidant Potential

Because of the extreme reactivity of hydroxyl radicals they are primarily analysed using radical trapping agents, followed by HPLC determinations of the reaction products. The radical trapping (or “spin trapping”) process allows free radicals to be investigated by transforming them into more stable species. When this method is used for biological analyses the radical trap agent should be selected extremely carefully to avoid toxicity problems. Examples of suitable radical trapping reagents are phenylalanine (with which hydroxyl free radicals react to produce tyrosines), derivatives of aspirin (*o*-acetylsalicylic acid) [2, 10]. It turned out that when *p*-hydroxybenzoic acid was used as a spin trap, the single reaction product 3,4-dihydroxybenzoic acid is completely separated from spin trap agent [12]. They can be separated using reversed phase HPLC or ion exclusion chromatography and detected photometrically or more sensitively by electrochemical detection using a glassy carbon electrode at 0.8 V vs Ag/AgCl [11].

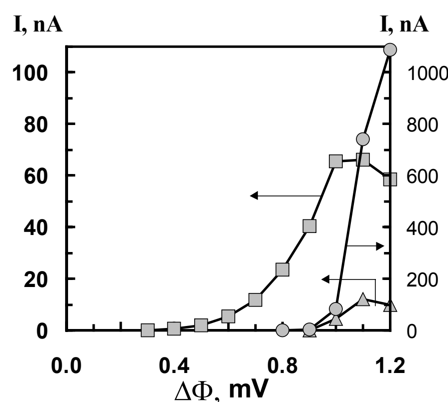


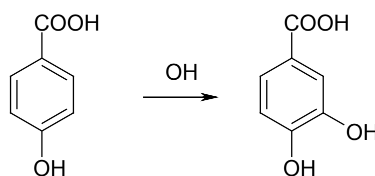
Fig. 5. Hydrodynamic voltammograms of 10 μmol L^{-1} 3,4- dihydroxybenzoic (squares), 10 μmol L^{-1} (triangles) and 1 mmol L^{-1} p-hydroxybenzoic acids (circles). Electrochemical detector +0.8 V vs Ag/AgCl. Chromatographic conditions: column - 300x7.8 mm I.D. TSK-GEL SCX(H^+) (TosoHaas); mobile phase - 1 mmol L^{-1} H_2SO_4, 1 mmol L^{-1} KCl, 0.125 mmol L^{-1} EDTA, 13% ACN; electrochemical detector +1.0 V vs Ag/AgCl; temp. - 20°C, flow rate – 0.9 mL min^{-1}

The method described above has been employed to the determination of the ability to scavenge OH radicals by various substances referred to as their "antioxidant capacity". In this assay reaction mixture contains both the substance tested and the "detector" spin-trapping agent - *p*-hydroxybenzoic acid. Hydroxyl radicals are generated by Fenton reaction and both the detector and the analyte scavenge the radicals. If the analyte "performs better" than the detector, generation of the dihydroxybenzoic acid is decreased. This assay enables to compare the OH radical scavenging performance of various substances, measured as a decrease of peak height of 3,4-dihydroxybenzoic acid. Hydrodynamic woltamograms obtained for 3,4-DHBA and *p*HBA are presented on Fig. 5. Decrease of the 3,4 DHBA peak means competition between *p*HBA and dopamine in reaction with hydroxyl radicals. In the other words it means that dopamine in scavenger of hydroxyl radicals.

It was found that, contrary to the photometric measurements, chromatographic measurements of TAP are not characterized by the linear calibration curve [26]. It is caused by the fact that in this case we measure decrease of the chromatographic peak. When it completely disappear further increase of the sample concentration gives no effect. Therefore, it is convenient to present results on the scale between 0 (lack of the interaction between radical and sample) and 1 (complete scavenge of free radicals by the sample).

Hydroxyl radicals were generated through Fenton reaction (1-3) by 1 min incubation of 0.5 mM Fe^{2+}, 2 mM ADP, and 2 mM H_2O_2 in 50 mM phosphate buffer (pH 7.4) in the presence of 1 mM *p*-hydroxybenzoic acid and analyzed phenylacetic acid at 37 ºC. Product of the reaction of *p*-hydroxybenzoic acid with hydroxyl radical:

*p*HBA + $^{\cdot}$OH = 3,4 DHBA



3,4dihydroxybenzoic acid, has been analyzed chromatographically. The reaction was stopped by 2 mM dimethyl sulfoxide (DMSO) and 0.1 mg/ml Desferal, and the reaction mixture was immediately analyzed by IEC.

Compounds containing phenolic rings (eg., flavonoids, salicylates) frequently display strong free radical scavenging properties. If such compound is biologically or pharmacologically active through mechanisms involving free radicals, free radical scavenging may modulate its activity.

On Fig. 6 the heights of chromatographic peaks obtained for some organic solvents (methanol, ethanol and dimethyl sulfoxide (DMSO)) are presented compared to the control (no analyte). These solvents are thought to be relatively effective hydroxyl radical scavengers, which property may be responsible for some protective activity of DMSO observed during oxidative stress in brain [27, 28]. Our results confirm that all these solvents possess antioxidant capacity, which is particularly high in the case of DMSO. The decrease in chromatographic

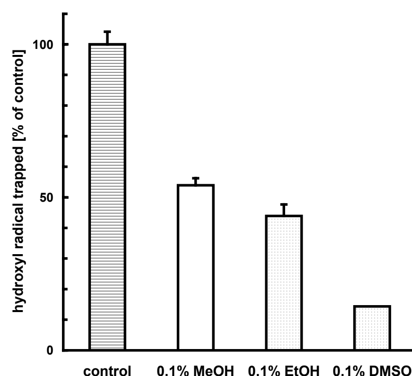


Fig. 6. Antioxidant capacity of methanol, ethanol and dimethyl sulfoxide. Hydroxyl radicals were generated using ($ADP/Fe(II)/H_2O_{2,}$) system, and trapped with p-hydroxybenzoic acid in the presence of the solvents tested at 0.1% concentration. Reaction product (3,4-dihydroxybenzoic acid) was assayed using ion-exclusion chromatography. The control sample contained no analyte, therefore the height of the reaction product with the detector substance was 100%. Chromatographic conditions as on Figure 5. Data presented as means ± SD.

peak height of dihydroxybenzoate is proportional to the analyte concentration, but it also is characteristic for each analyte, which probably is dependent on the rate constant of the reaction with hydroxyl radicals. These rate constants equal 8.3×10^8, 2.2×10^9 and 7.0×10^9 mol $L^{-1}s^{-1}$ for methanol, ethanol and DMSO, respectively [29, 30].

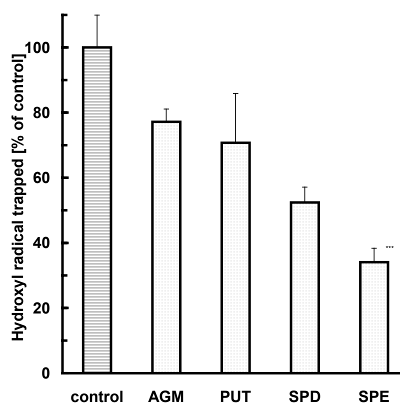


Fig. 7. Antioxidant capacity of biogenic polyamines (AGM – agmatine, PUT – putrescine, SPD – spermidine and SPE –spermine).

On Fig. 7 the heights of chromatographic peaks obtained for biogenic polyamines putrescine, spermidine, spermine and agmatine are presented. These polyamines occur in virtually all living organisms [31]. They are essential in cell growth and replication functions. They also are binding antagonists of some common cations like K^+, Mg^{2+} or Ca^{2+} and putative

epidermal antioxidants. Changes of their concentration are observed during reperfusion after short ischemia. It remains controversial whether their production has neurotoxic or neuroprotective effects. Our results indicate that these amines are able to scavenge OH radicals. Similarly to the case of organic solvents, it appeared that antioxidant potential of polyamines was roughly proportional to their rate constants with hydroxyl radical, which for putrescine, spermidine and spermine equal 1.1 x 10^8, 1.2 x 10^8 and 1.3 x 10^8 mol $L^{-1}s^{-1}$, respectively [10].

Phenylacetate (PA) and phenylbutyrate (PB) are phenolic ring-containing cell differentiating compounds, which may also induce apoptosis of malignant cells [11]. Their radiosensitizing properties and additive or synergistic interactions with other cytotoxic drugs have also been demonstrated. Unlike most anticancer drugs which are active in nano-to-micromolar concentrations, PA and PB exert *in vitro* and *in vivo* anticancer activities only at relatively high concentrations (> 0.5 mM in cell cultrure media or plasma). Nevertheless, they are fairly well tolerated; in particular they are devoid of bone marrow toxicity [7]. These compounds are currently under development as monotherapy of refractory malignancies. Their use in combination with other cytotoxic agents or radiotherapy may be considered on the basis of the aforementioned in vitro data. It turned out that the investigated acids are weaker scavengers of hydroxyl radicals, compared to polyamines or DMSO [11]. Similar results were obtained using TRAP method. Presented data indicate that phenylacetates, inspite being cell differentiation or apoptosis inducers, display properties of weak free radical scavengers. Considering that these compounds provoke cell differentiation only in relatively high (submilimolar-to-milimolar) concentrations, one may expect that, at the same time, intracellular antioxidative potential is substantially reinforced. It remains to be shown whether phenylacetates induce intracellular oxidative stress or they represent a class of differentiation and apoptosis inducers, which act without the involvement of oxidative stress.

In the literature some studies indicated that catecholamines (among them dopamine and L-dopa) may be pro-oxidants as well as antioxidants [32, 33]. Therefore, changes of free radicals concentration and, indirectly, total antioxidant potential (TAP) of blood plasma of PD patients should be expected. Additionally uptake of L-dopa decreases characteristic for PD tremor, which leads to hypermetabolism and increase FR generation.

Also in the literature we can find contradictory information about oxidative properties of dopamine. From the one side dopamine is involved in oxidative stress (because of catalyze of Fenton reaction by reduction of transition metal ions and generation of semiquinone and superoxide anion radicals during their reduction), from the other acts as an antioxidant (because of their ability to scavenge free radicals by catechol and aromatic groups and complexation of transition metal ions).

It turned out that DA decreased 3,4-DHBA peak. It means that in the presence of strong antioxidant (ADP) DA scavenge hydroxyl radicals. However, in the absence of ADP in the reaction mixture DA dopamine increases peak of 3,4-DHBA. It means that in this case DA is pro-oxidant, probably because it reduces iron ions, which catalyze Fenton reaction. Similar results were obtained for L-dopa. This result means that depending on the concentration and the presence in the solutions other antioxidants, DA can be pro- or antioxidant.

Enzymatic oxidation of dopamine by MAO B and its auto-oxidation produce hydroxyl peroxide (H_2O_2). Although H_2O_2 is not reactive compound, however, in Fenton reaction

(DA promote it because of its ability to reduce Fe(III) to Fe(II), which catalyzes reaction), it generates extremely reactive hydroxyl radicals. Additionally, metabolism of DA produces 6-hydroxydopamine and semiquinone radical, which are oxidants. Therefore, it can damage neurons causing even their death by apoptosis.

From the other hand catechol group in DA molecule is characterized by strong antioxidant properties. Additionally, DA complex Fe(II), therefore, preventing Fenton reaction. We can see that influence of DA on free radicals is rather complicated and its pro- or antioxidative properties depend on its concentration.

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FRACTIONATION AND IDENTIFICATION OF UNDERIVATIZED POSITIONAL AND GEOMETRIC ISOMERS OF CONJUGATED LINOLEIC ACID

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Key words: conjugated linoleic acid isomers, separation, identification, silver-ion HPLC

Introduction

Conjugated linoleic acid (CLA) isomers are a group of (*n-6*) polyunsaturated fatty acids found in ruminant fats and is said to be comprised at least of eight isoforms. CLA isomers are physiologically highly active compounds that differ in their biologic properties in several ways from linoleic acid. Many studies on laboratory animals reported differences in the nutritional and physiological functions of various geometric and positional isomers of CLA (1). Considering the growing demands for investigation of physiological properties of individual isoforms of CLA and their metabolites, new strategies can be tried to improve determination of conjugated fatty acids (CFA) from specimens of animal and food product origin. The aim of the study was developing the more versatile liquid chromatographic (HPLC) method with photodiode detection (DAD) for optimal quantification and distingui-shing of *trans,trans, cis,cis* and *cis,trans/trans,cis* isomers of CLA. Several investigators detected changes in the CLA isomer profile (2) due to the use of heat and acid during saponi-fication and derivatization, thus we used mild conditions of saponification (low temperature and minimal base concentration) for preparation of free fatty acids hydrolysates (3). Finally, the silver-ion chromatography with DAD for quantification of free CFA was applied.

Experimental

Saponification: The fine powdered rats' liver or muscle sample (~50 mg) was treated with a mixture of 2 ml of 2M KOH in water, 2 ml 1M KOH in methanol, then flushed with stream of argon for ~3 min. The resulting mixture in a closed vial was then vigorous mixed and heated under argon at 95°C for 10 min, next cooled for 10 min at the room temperature, and, then, sonicated for 10 min. Obtained solution was protected from the light and stored in a sealed vial at 22–25°C for overnight.

Extraction of free FAs: To the hydrolysates in vials 3 ml of water were added and the resulting solution was than vigorous mixed. The obtained solution was acidified with 4M HCl to pH 1-2 and then free FAs were extracted four times with 3 ml of dichloromethane (DCM). The lower DCM layer was dried with ~0.1 g of Na_2SO_4. To avoid any loss of free FAs extraction was repeated using 4 times 3 ml portion of heptane. Afterward, the heptane layer was combined with DCM layer and then organic solvents were removed under argon. Finally, the residue was

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re-dissolved in 1 ml of DCM and then 20-40 µl of the resulting solution were injected onto the silver-impregnated columns.

Silver-ion chromatography (Ag^+-HPLC): Fractionations of free CFA were carried out using isocratic chromatography (two 250 x 4.6 mm Chrompack ChromSpher 5 µm Lipids columns) and the photodiode array detector (Waters) operated in the UV range from 195 to 360 nm with a spectral resolution of 1.2 nm and a measurement frequency of 1 spectrum/sec. All runs were performed at a flow-rate of 1.35 ml/min, while a column heater was maintained the temperature at 25°C. The eluent for the elution was prepared from heptane (95%; Lab-Scan), acetic acid, acetonitrile (99.483:0.5:0.017, v/v/v). The silver-impregnated columns were equilibrated with the freshly prepared mobile phase at least 20 min before sample injection.

Results and discussion

Free CLA isomers, like other conjugated fatty acids, had high molar absorptivity at ~234 nm because of the presence of a chromophore group (i.e. double bonds separated by a single carbon-carbon bond-conjugated unsaturated (2-4). The high molar absorptivity and the close proximity of the absorbance maximum to 234 nm make free CLA isomers as well as all other CFAs almost ideally suited for the highly efficient Ag^+-HPLC separation with direct UV detection at ~234 nm. Very low background fluctuation was observed and all peaks were absent from the blank, when the current chromatographic procedure was applied. The nature of the Ag^+-HPLC separation obtained with the standard mixture of the CLA isomers (Sigma, USA) is illustrated in Fig. 1. Our current chromatographic method allows better fractionation of geometric the CLA isomers, as well as all positional isomers within each geometric group of CLA isomers (Fig. 1) compared with our previous gas chromatography techniques and reversed-phase C_{18}-HPLC methods (3,5). Moreover, proposed isocratic elution system composed of acetic acid (0.5%) and ACN (0.017%) in heptane, a flow-rate of 1.35 ml/min and UV detection in at 234 nm were found to provide excellent baseline stability. What is particularly puzzling is that heptane only with the higher concentration of ACN compared with the concentration of ACN in the mobile phase containing n-hexane (4) ensured very small baseline noise and substantial retention of all assayed CLA isomers on silver-impregnated columns. The addition of the greater amount of ACN (from 0.017 to 0.02%) to heptane permitted excellent peak shapes without "tailing", close to symmetrical even with elution times from 25 to 60 min, hence, excellent resolution of all CLA isomer peaks was obtained. Interestingly, as can be seen from UV spectra (Fig. 1), the absorption spectra of *trans-trans, cis-trans/trans-cis* and *cis-cis* CLA isomers bear a close resemblance, however, the absorbance maximum evidently depended upon the geometric form of assayed isomers (at 231.9, 234.3 and 235.4 nm). Considering the above results, the use of DAD to identify the geometric configuration of the CLA isomers can be recommended.

We found that the resolution of the overlapping peaks, especially of the most abundant CLA isomers and unidentified their metabolites can be improved by using dichloromethane (DCM) as a solvent applied for re-dissolving residues obtained after drying of hydrolysates under the stream of argon. Moreover, only DCM (contrary to methanol, hexane or heptane) permitted satisfactory solubility of these residues from saponified specimens of animal origin (e.g. liver, muscles or blood serum). As the presented chromatographic method

was applied to biological samples (blood serum, rats liver or muscles), very small fluctuation of background, the satisfactory sensitivity and resolution of all geometrical and positional isomers of CLA and their metabolites can be obtained (Fig. 2).

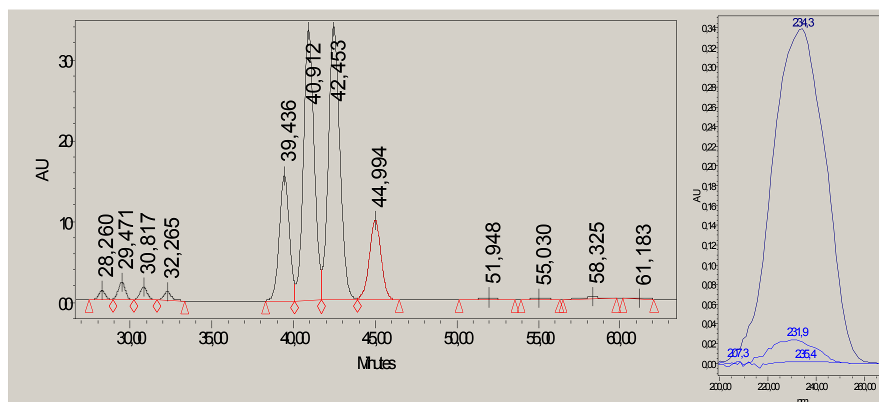

Fig. 1. Separation of a free CLA isomer mixture (Sigma) by HPLC in the silver-ion mode (the left part of the figure); UV detection at 234 nm. Individual CLA isomers - from 28 to 33 min: *trans-11trans-13, trans-10trans-12, trans-9trans-11, trans-8trans-10;* from 39 to 45 min: *cis-11trans-13/trans-11cis-13, cis-10trans-12/trans-10cis-12, cis-9trans-11/trans-9cis-11, cis-8trans-10/trans-8 cis-10;* from 51 to 62 min: *cis-11cis-13, cis-10cis-12, cis-9cis-11, cis-8cis-10.* The right part of the figure: UV absorbance spectra of groups of geometric CLA isomers: *trans,trans* (the absorbance maximum at 231.9 nm), *cis,trans/trans,cis* isomers (the absorbance maximum at 234.3 nm) and *cis,cis* (the absorbance maximum at 235.4 nm).

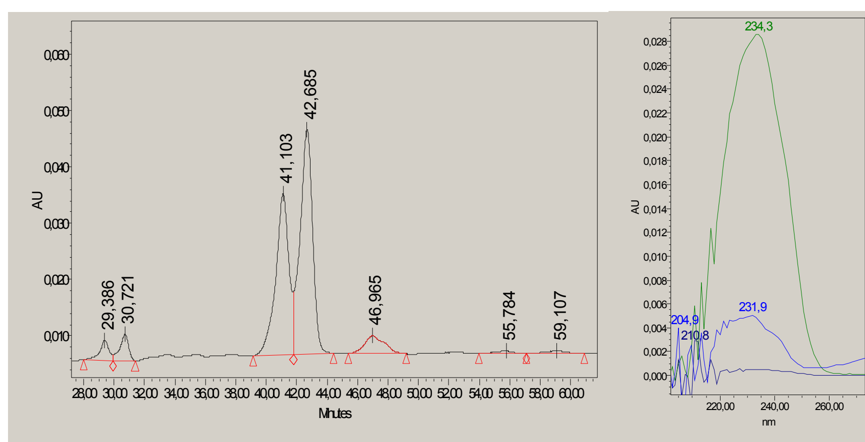

Fig. 2. A typcal chromatogram of blood serum of rats fed the diet enriched in 2% CLA isomer mixture. The identified isomers: from 29 to 31 min – *trans,trans*CLA (the absorbance maxi-mum at 231.9 nm); from 41 to 43 min – *trans-10cis-12*CLA, *cis-9trans-11*CLA (the absorbance maximum at 234.3 nm); at 46.965 min – a metabolite of CLA isomer (the absorbance maxi-mum at 234.4 nm); from 51 to 62 min – *cis,cis*CLA isomers (the absorbance maximum at 235.4 nm)

Conclusion

Direct injection of mildly saponified lipids to free FAs is to be the most accurate chromato-graphic method of quantifying CFAs. Indeed, recent studies show the difference in the composition of all geometric and positional isomers of CFAs resulting from typically used methylation methods (due to *intra*- or/and *inter*-isomerisation). Therefore, direct high resolution silver-ion chromatography based on photodiode detection should aid in understanding the mechanism responsible for diverse physiological functions reported for CLA isomers and their metabolites, due to more confidential measurement of free fatty acids containing conjugated double bonds distributions. Large samples of animal origin can be analysed since saponified samples were dissolved in dichloromethane. Therefore satisfactory accuracy, precision and sensitivity (the limit detection: 0.1–1.0 ng/l (1)) can be achieved for conjugated fatty acids in assayed blood plasma, muscles or liver of rats or sheep.

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THE EFFECT OF BUTYLATED HYDROXYTOLUENE ON COMPOSITION OF METHYLATED FATTY ACIDS ASSAYED BY GAS CHROMATOGRAPHY

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Introduction

Recently, we have demonstrated that capillary gas liquid chromatography (GLC) and silver-liquid chromatography (Ag^{+}-HPLC) may be adapted to analyze a mixture of fatty acids containing conjugated double bonds (e.g. CLA isomers) as well as unsaturated and saturated fatty acids (Czauderna et al., 2005). The reproducibility of assay of fatty acids (FAs) was satisfactory sensitive to small changes in temperature of processing samples, presence of endogenous substances in biological materials and the content of pro-oxidative species as well as antioxidant. The concentration of pro-oxidative and antioxidant species resulted in significantly affected the profile of particularly polyunsaturated fatty acids. Unfortunately, polyunsaturated fatty acids (PUFA) are particularly sensitive to oxidation in comparison to monounsaturated (MUFA) or saturated (SFA) fatty acids in assayed biological samples. Especially, oxidation and interisomerization during saponification, extraction and derivatization resulting in inaccurate MUFA and PUFA determination. Therefore for optimal quantification, the se-lection of FAs protection method against oxidation is crucial to successfully quantifing fatty acids such as particularly MUFA and PUFA including CLA isomers. In current study, two methods have been compared which involve the use of butylated hydroxytoluene (BHT) (Method BHT) or argon (Method Ar) to remove of air from processing samples.

Experimental

In this present study, a commercial mixture of CLA isomers (Larodan; purity ~95 %) (Czauderna et al., 2003), saturated and unsaturated fatty acid methyl esters (Supelco) and lyophilized rat' liver were used to evaluate the influence of Ar and BHT on the profile of assayed FAs after saponification, extraction and methylation of extracted free FAs.

Methods Ar and BHT: The first step procedure was mild saponification (Czauderna at al., 2005) of a CLA isomer mixture (~8.9 mg), unsaturated fatty acid methyl esters (~5.5 mg) and a lyophilized liver sample (~50 mg of DM) (Czauderna et al., 2004). Assayed samples were treated with a mixture of 2 ml of 2 M KOH in water, 2 ml 1 M KOH in methanol flushed with a stream of Ar for 4-5 min (Method Ar) or treated 50 μl of BHT solution in methanol (20 mg/ml) and next flushed with a stream of Ar for 3 min (Method BHT). The resulting mixture in a tightly closed vial was then vigorous mixed and heat at 95°C for 10 min, next cooled for 10 min at the room temperature and, then, sonicated for 10 min. Obtained solution should be protected from the light and stored in a sealed vial at ~22°C for overnight.

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Extraction of free FAs: To the hydrolysatese in a vial 3 ml of water were added and the re-sulting solution was than vigorous mixed. The obtained solution was acidified with 4 M HCl to pH 1-2 and then free FAs were extracted four times with 3 ml of dichloromethane (DCM). The lower DCM layer was dried with ~0.1g of Na_2SO_4. To avoid any loss of free FAs extraction was repeated using 4 times 3 ml of *n*-hexane. Afterward, the upper *n*-hexane layer was combined with DCM layer and then organic solvents were removed under a stream of Ar.

Preparation of fatty acid methyl esters (FA-MEs): To the residue was added 2 ml of 2 M NaOH in methanol, and next flushed with a stream of Ar for 4-5 min (Method Ar) or treated with 50 µl of BHT solution in methanol (20 mg/ml), and then flushed with Ar for 3 min (Method BHT), and next reacted for 1 h at 80°C. After cooling to reaction mixture 2 ml of 25 % BF_3 in methanol were added, flushed with Ar for 3 min (Method BHT) or for 4-5 min (Method Ar), and again heated for 1 h at 80°C. To a cooled reaction mixture was added 5 ml of water and then FA-MEs were extracted with 5 ml of n-hexane. The clear supernatant was transferred to a vial. Separation of all FA-MEs was carried out using GLC (Czauderna et al., 2005), while FAs containing conjugated double bonds using also isocratic liquid chromato-graphy (Ag^{+}-HPLC) with photodiode array detection at 234 nm (Czauderna et al., 2005).

Results

Recently, we reported (Czauderna et al., 2005) successful separation of complex mixtures of FAs from the specimens of ruminant' origin on a commercially available a 100 m polar capillary column for GLC and a silver-impregnated column for HPLC. Therefore, we attempted first to analyse the mixture of the methylated fatty acids in standards and rats' liver using the gas chromatography and silver-ion liquid chromatography with DAD. The results presented in Table 1 show the difference in the composition of particularly unsaturated fatty acids present in the commercial FAs mixture as well as in assayed liver. Unexpectedly, Method Ar was associated with increase the peak areas of all CLA isomers in comparison with analysis of the CLA isomer mixture was performed using Ag^{+}-HPLC (Czauderna et al., 2005) with ones obtained from analysis performed according to Method BHT. We hypothesized that this surprisingly results is probably due to isomerization of some components (e.g. linoleic acid) in a commercial CLA isomer mixture and next formation of extra species possessing conjugated double bonds during in hydrolysis and base-acid catalyzed-methylation in the solution in the absence of BHT (i.e. Method Ar). Detailed analysis of other non-conjugated FA standards revealed that in the presence of BHT the concentration of methylated fatty acids are higher in comparison with ones in solutions flushed only with a stream of Ar (Method Ar). In the liver sample investigated (Table 1), the concentration of all assayed fatty acids (with the exception of C22:5n-3) was also higher in the processed solutions in the presence of BHT (Method BHT) than in solutions flushed with Ar (with the exception of C22:5n-3). There is no clear explanation for such the strange concentrations of C22:5n-3, but it may be suggests that in the absence of BHT the unidentified specie(s) co-eluted with C22:5n-3 has/have been formed during saponification or/and derivatization of free FAs.

Table 1. Comparison of the effect of different methods on the peak area (S_n) of assayed fatty acids in a rat' liver and in the standard mixed fatty acids

Assayed methylated fatty acids	Method Ar S_n	Method BHT S_n
Processed standard mixed fatty acids		
*cis-9,trans-11*CLA	226	217
*trans-10,cis-12*CLA	182	117
*cis,cis*CLA	241	172
*trans,trans*CLA	240	218
Sum of all CLA isomer[1]	$31.9\cdot10^6$	$28.6\cdot10^6$
C16:0	114	111
C18:0	137	125
*cis-9*C18:1	105	158
*cis-9,cis-12*C18:2	82	105
*cis-9,cis-12,cis-15*C18:3	75	93
*cis-11*C20:1	61	79
*cis-11,cis-14*C20:2	40.5	53
*cis-11,cis-14,cis-17*C20:3	20.5	26
*cis-5,cis-8,cis-11,cis-14*C20:4	9.4	11.0
*cis-5,cis-8,cis-11,cis-14,cis-17*C20:5	3.5	5.5
Processed rat' liver		
C16:0	141	157
C18:0	191	237
*cis-9*C18:1	39.2	41.5
*cis-9,cis-12*C18:2	84	88
*cis-9,cis-12,cis-15*C18:3	9.0	9.7
*cis-7,cis-10,cis-13,cis-16,cis-19*C22:5	54.1	1.7
*cis-4,cis-7,cis-10,cis-13,cis-16,cis-19*C22:6	3.8	15.3

Conclusions

The current study provides the universal method for gentle saponification and next the base-acid catalyzed methylation of FAs in obtained hydrolysates. The results show that the saponification and derivatization in the presence of BHT (Method BHT) is to be the more accurate method for quantifying FAs in a commercial standards and biological samples. However, the influence of BHA as well as other anti-oxidants on the accuracy of FAs assays should be studied further.

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REVERSED-PHASE LIQUID CHROMATOGRAPHY FOR FREE AND PROTEIN AMINO ACIDS FROM SPECIMENS OF ANIMAL ORIGIN*

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Key words: amino acids, seleno-amino acids, reversed-phase HPLC

Introduction

The ability to separate and quantitate amino acids (AAs) has many applications in the areas of clinical chemistry and protein chemistry (1,2). AA analyses are usually accomplished by separating AA mixtures applying ion-exchange chromatography followed by *post*–column derivatization with ninhydrin or fluoregenic reagents. These procedures resolve most AAs with good detection limits, particularly if *o*-phthaldialdehyde (OPA) is applied as the *post*-column derivatizing reagent. Unfortunately, *post*-column techniques mostly require specialized and high costly equipment dedicated only to AA analysis. Therefore, it seems to be useful to develop the simple reversed-phase (RP) HPLC procedure with *pre*-column deri-vatization for the analysis of protein- and free AAs in physiological fluids as a cheaper alternative to commercial amino acid analyzers. The aim of this study was to develop new gradient conditions for simultaneous analysis of typical amino acids as well as rare amino acids like seleno-cystine (Se-Cys), seleno-methionine (Se-Met), 2,6-diaminopimelic acid (DAPA) or homo-cystine (h-Cys) in physiological fluids. Indeed, there is growing interest in particularly Se-Cys, Se-Met and h-Cys assays, considering beneficial in cancer and heart diseases prevention. Fortunately, the OPA derivatization is suitable for automation, it seems to be useful to compile the *pre*-column OPA-derivatization procedure with high-resolution C_{18}-chromatographic separation, fluorescence and photodiode array detections. Because of the complexity of the biological materials, high-resolution liquid chromatography with sensitive and selective detection is necessary. In order to improve selectivity, a RP long column containing dimethyloctadecylsilyl-bonded amorphous silica is to be used. The main purpose of the current work was to find the more versatile liquid chromatographic method with detection and/or fluorescent detection than previously published (3,4) for free amino acids, particularly Se-Cys, Se-Met and h-Cys in blood serum of animals. In addition, attention has been paid to adapt the C_{18}-liquid chromatography for simultaneous determination of Se-Cys, Se-Met and h-Cys together with other free AAs in blood serum of animals.

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Experimental

All reagents were of analytical grade; methanol and tetrahydrofuran (THF) and water were HPLC grade. Methanol and THF were purchased from POCh (Poland). Etanothiol (ESH), *o*-phthaldialdehyde (OPA) and all amino acid standards used were from Sigma (USA). The buffers for mobile phases were filtered though a 0.45 μm membrane filter (Millipore).

For the separation of derivatized amino acids (AAs) an 2690 Alliance separation module with a Waters 996 photodiode array detector (DAD) and a Waters 474 fluorescence detector operated at the gain 10. Derivatized AAs in the effluent were monitored using DAD operated in a UV range from 195 to 400 nm with a spectral resolution of 1.2 nm and a measurement frequency of 1 spectrum/sec. Fluorescence detections were taken at the optimum excitation and emission wavelengths at $\lambda_{ex}/\lambda_{em}$=231/470 nm (FD-1) and $\lambda_{ex}/\lambda_{em}$= 336/425 nm (FD-2). An analytical column used was the Nova-Pac C_{18} column (4 μm, 250x4.6 mm, Waters) in conjunction with a guard column. All separations were performed at a column temperature of 37°C. For analysis of derivatized AAs in all assayed biological samples, the quaternary gradient elution program was used (Table 1). The solvent A was THF and the buffer I (1:99, v/v). The buffer I was prepared from 0.02 M Na_2HPO_4 adjusted to pH 3.5. The solvent B was THF and the buffer II (1:99, v/v); the buffer II was prepared from 0.04 M Na_2HPO_4 adjusted to pH 6.6. The solvents C and D were methanol and water, respectively. AAs in assayed biological samples were identified by the retention time of AA standards injected separately and by adding of processed AAs to biological samples.

Table 1. Quaternary gradient elution program used for analysis of free and protein amino acid derivatives (OPA-AAs) in all assayed biological samples (the flow-rate 1.8 ml/min)

Time (min)	Composition[a] (%)			
	Solvent A	Solvent B	Solvent C	Solvent D
0	0	85	15	0
1.8	0	85	15	0
3.0	0	72	28	0
18.8	0	72	28	0
19.0	0	60	40	0
21.0	0	64	36	0
23.0	0	54	46	0
27.0	18	54	28	0
29.2	19.5	68.5	12	0
29.5	48	0	52	0
37.5	43	0	57	0
39.0	0	44	56	0
44.0	0	37	63	0
47.0	0	40	60	0
48.5	0	35	65	0
49.5	0	45	55	0
60.0[b]	0	0	85	15

[a] all changes of solvents composition were linear

[b] after 70 min, the column was re-equilibrated for 10 min in 85% solvent B and 15% solvent C borate buffer (pH 9.9). Next, 70 μl ESH were added and the resulting solution was mixed. The reagent solution was prepared at least 2 h before use. The reagent strength was maintained by addition of 10 μl of ESH every 2-3 days.

Preparation of derivatizing reagent (OPA/ESH) and samples for HPLC analysis

Preparation of OPA/ESH: 75 mg of OPA were dissolved in 4.5 ml methanol and 0.5 ml 0.4 M

Preparation and hydrolysis of biological samples with 6 M HCl: Femoral muscles and livers were collected from female rats (8 weeks of age; Wistar) (4). Obtained samples were freeze-dried. Then, obtained homogeneous materials (~0.5 g) were hydrolyzed with 50 ml of 6 M HCl at 104±2°C for 20 h in sealed tubes. After cooling the hydrolysates were filtered through filter paper and washed three times with water. Then HCl was removed from the filtrates. Ten ml water were added to the residue and then evaporated to dryness again in vacuum to remove residues of HCl. This evaporating procedure was repeated twice. The residue was re-dissolved in 1 ml of the borate buffer (pH 9.9). 10-40 μl of resulting solution was used for the OPA/ESH derivatization procedure as below.

Preparation of ovine and rat blood serum for free amino acid HPLC analyses: Blood samples from rats and sheep were collected into heparinized tubes (kept in an ice bath) and centrifuged at 2000 *g* for 15 min (at 0-4°C). Blood serum was stored at -29°C. On the day of analysis, 1 ml of serum (0-1°C) was deproteinized with 1 ml of 7% cooled solution (0-1°C) of trichloroacetic acid and centrifuged at 2000 *g* for 15 min (at 0-4°C). The obtained supernatant was filtered through a 0.2 μm nylon filter (Cole Parlmers) into an autosampler vial. 50-100 μl of supernatant was used for the OPA/ESH derivatization procedure as below.

Derivatization procedure: To an autosampler vial were added an appropriate volume (20-100 μl) of assayed biological sample, 0.5 ml of OPA/ESA derivatizing reagent and 10-30 μl of 1 M NaOH. The content was mixed and reacted for 3 min at room temperature. The pH of the resulting solution should be from 9 to 10. At the end of the 3 min derivatization period, the processed samples were injected onto the column, so, the total time of the reaction was ~4 min. The derivatizing procedure for standards was the same as for biological samples. It is re-commended to protect all derivatized amino acids (OPA-AAs) from the light and to store them at -29°C until analyzed.

Results and discussion

Although the liquid chromatography of OPA-AAs has been recently reported, the fractionation of complex mixture of free amino acids as well as protein hydrolyzates necessitated development of a new gradient program and detection methods that would be exhibit an improvement in accuracy, precision, selectivity and detection limits. Detailed investigation of various organic solvent mixtures (acetonitrile, methanol and THF) and buffers having various pH showed that methanol, THF and two buffers adjusted to pH 3.5 and 6.6 and proposed column temperature enabled the optimum separation conditions of all assayed amino acids using UV and fluorescence detections (FD-1 and FD-2). The proposed HPLC procedure resulted in system pressure changes (from 25 to 36 MPa) in accordance with chromatographic rules, excellent OPA-AAs peak shapes, close to symmetrical even with elution times to 61 min and such background fluctuations witch permitted accurate and precise quantitate all OPA-AAs. OPA-AAs peaks were differentiated from unidentified species in all assayed biological samples

by the use DAD detector as well as fluorescence monitoring. No co-elution of OPA-AAs with unindentified endogenous species in all examined specimen of biological origin was observed for DAD monitoring (in the spectral range of 205–370 nm) and also FD-1 and FD-2. The accuracy of OPA-AAs assay and purity was investigated also in details by comparison UV-DAD spectra of OPA-AAs of all assayed biological samples with UV-DAD spectra of OPA-AAs in processed standards. Considering the results of UV-spectra comparison it is reasonable to conclude that all OPA-AAs peaks were pure (in the UV range of 212–370 nm) and, so, avoid co-elution with endogenous species in examined samples (liver and muscles from rats and serum from sheep and rats). All OPA-AAs peaks were absent from the blank, when the current HPLC procedure was applied.

Table 2. Relationship between the wavelength (λ=231 nm *vs.* λ=336 nm) of OPA-AAs UV-detection, the mode of fluorescence detection (FD-1 and FD-2) and stability of OPA-AAs with respect to storage time of processed sample at -20°C; ratios (R) – after 80 min and 24 h[8]

Amino Acid	Ratio of UV/FD[1]	UV detection					Fluorescence detection				
		231[2] / 336	storage: 80 min[3]		Storage: 24h[3]		FD-1[4] / FD-2	storage: 80 min[3]		storage: 24h[3]	
			R_{231nm}	R_{336nm}	R_{231nm}	R_{336nm}		R_{FD-1}	R_{FD-2}	R_{FD-1}	R_{FD-2}
K-asp	4.24	7.42	1.01	1.00	1.18	0.83	1.67	0.93	0.99	0.86	0.82
K-glu	3.72	7.59	0.97	1.22	1.25	0.83	1.14	0.99	1.00	0.94	0.82
Asp	2.70	5.84	1.01	1.00	0.94	0.81	1.00	0.99	0.98	0.93	0.81
Glu	2.41	5.68	0.99	1.01	0.92	0.96	0.84	0.96	0.86	0.86	0.94
His	2.77	5.79	1.00	1.01	0.96	0.90	1.06	0.98	0.95	0.94	0.91
Ser	2.62	6.03	1.01	1.02	0.96	0.76	1.08	0.99	0.99	0.95	0.72
Arg	2.42	5.77	0.96	1.01	0.96	0.82	1.02	0.99	1.01	0.96	0.88
Gli	2.50	5.55	0.99	1.03	1.21	1.06	1.01	0.98	1.00	1.12	1.07
Tre	2.82	5.70	0.98	1.04	1.00	0.70	1.17	1.00	1.15	0.94	0.70
Tyr	2.43	5.88	1.00	1.00	0.76	0.84	1.25	1.00	0.90	0.91	0.80
Ala	1.69	5.41	1.00	0.98	0.86	0.92	0.68	0.98	1.00	1.07	0.84
Taurine	3.36	5.20	0.99	0.99	0.84	0.60	1.04	1.02	1.00	1.01	0.78
Met	2.20	5.93	1.00	1.01	0.91	0.81	0.99	0.99	1.00	0.93	0.81
Val	2.09	5.60	1.00	1.00	0.93	0.83	1.08	0.99	0.99	0.91	0.84
Se-Met	2.14	5.78	1.00	0.99	0.94	0.88	0.96	0.99	0.99	0.93	0.84
P-ala	2.06	5.47	1.00	1.00	0.98	0.80	1.08	0.99	1.00	0.88	0.80
DAPA	5.30	5.48	1.00	1.01	0.97	0.82	0.85	0.99	1.06	0.92	0.81
i-Leu	2.28	6.00	1.00	0.99	0.99	0.88	1.04	0.99	0.99	0.90	0.82
Leu	2.35	6.37	1.00	0.98	0.98	0.84	0.98	0.99	0.99	0.94	0.82
Se-Cys	-[5]	7.25	1.38	1.62	1.56	7.96	-[5]	-[5]	-[5]	-[7]	-[7]
Cys	61.88	6.39	1.05	1.08	-[7]	2.58	0.09	-[6]	0.95	-[7]	-[7]
h-Cys	12.66	5.46	1.12	1.12	-[7]	2.22	0.69	0.86	0.85	-[7]	-[7]
Lysine	3.27	5.54	1.01	1.00	1.11	0.86	0.65	1.00	1.00	1.08	0.80

[1]UV detection at 231 nm/fluorescence detection (at $\lambda_{ex}/\lambda_{em}$=336/425 nm); time reaction: 3 min
[2]the ratio (R) of OPA-AA peak areas obtained using λ=231nm and λ=336 nm, respectively
[3]the ratio (R) of OPA-AA peak areas detected after 3 min storage to 80 min and 24 h storage at -29°C
[4]the ratio of OPA-AA peak areas obtained using: $\lambda_{ex}/\lambda_{em}$=231/470nm (FD-1) and $\lambda_{ex}/\lambda_{em}$=336/425nm (FD-2)
[5]Se-cys – not detected using FD-1 as well as FD-2 modes
[6] Cys – not detected using FD-1 after 80min storage
[7] not detected after 24 h storage (i.e. below the limit of quantification due to the high level of a noise)
[8]ababbreviations for amino acids as in Fig. 1

Very good resolution of taurine and all AAs including glutamine, 2,6-diaminopimelic acid (DAPA), Se-Cys, Se-Met and h-Cys was obtained using new quaternary gradient elution program (Table 1) and UV and fluorescence detections. The typical UV-chromatograms at 336 and 231 nm showing the elution profile of derivatized AA standard are shown in Fig. 1 A, B, C and D. The attempts were also made to compare the results of OPA-AAs quantification as dependent upon used the type of the detector (UV detections vs. FD-1 and FD-2 detections). Detailed analysis of FD-chromatograms for all assayed OPA-AAs re-vealed that both fluorescence detection modes (FD-1 and FD-2) provide generally excellent low background under the analytical peaks as well as low fluctuation of baseline level from the left and right side of OPA-AA peaks. As can be observed from Fig. 1 A and B, the OPA-AAs measurement at 231 nm produced usually 5.2-7.6 greater signals than the UV detection at 336 nm (Table 2) as well as considerably higher response in comparison with usually used fluorescence detection of OPA-AAs ($\lambda_{ex}/\lambda_{em}$=336/425 nm; the gain=10). Moreover, the new gradient elution program assured simultaneously determination of DAPA, taurine and other AAs including Se-Cys and Se-Met by applying FD-1 and FD-2, and UV detections at 231 and 336 nm. So, our methodology should be also applicable to the analysis of marker of bacterial protein production and bacterial contamination of biological materials.

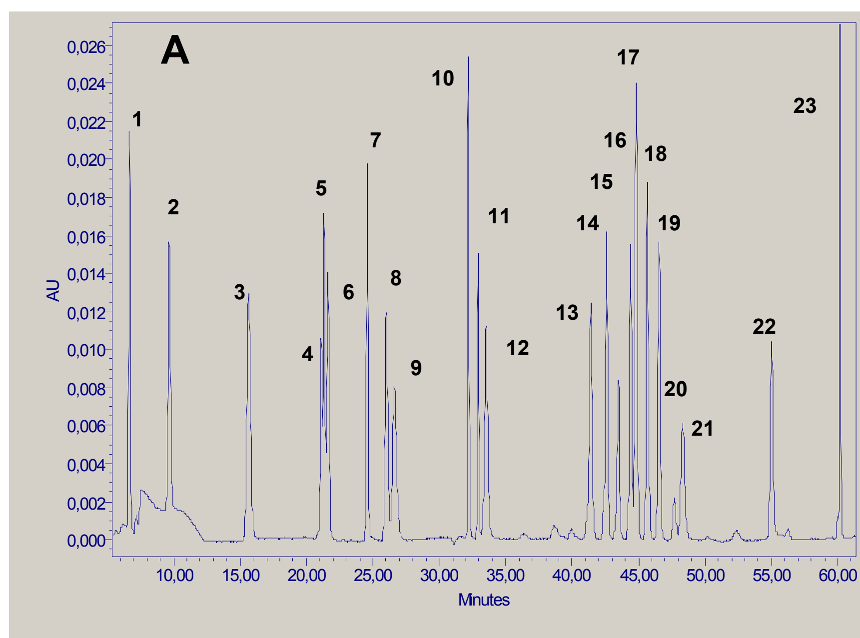

Fig. 1. Application of the gradient program and UV detection at 336 nm (A), for the standard of OPA amino acid derivatives: 1 - aspartic acid (K-asp); 2 – glutamic acid (K-glu); 3 – aspargine (Asp); 4 – glutamine (Glu); 5 – histidine (His); 6 – serine (Ser); 7 – arginine (Arg); 8 – glycine (Gli); 9 – threonine (Tre); 10 – tyrosine (Tyr); 11 – alanine (Ala); 12 - taurine; 13 – methionine; 14 – valine (Val); 15 – Se-Met; 16 – phenylalanine (P-ala); 17 – 2,6-diaminopime-lic acid (DAPA); 18 – *iso*-leucyne (*i*-leu); 19 – leucine (Leu); 20 – Se-Cys; 21 - Cys; 22 – homocystine; 23 – lysine (the procedure: 30µl AA standard, 0,5ml OPA and 15 µl 1 M NaOH)

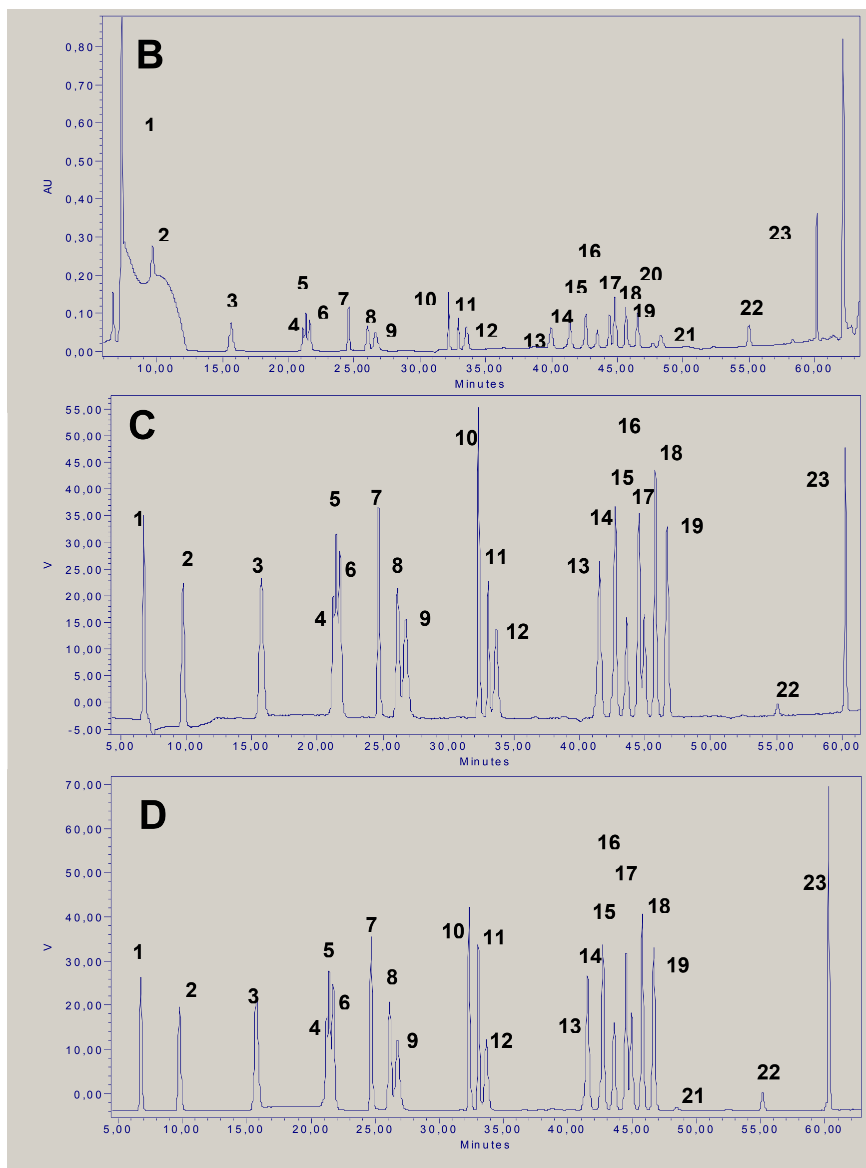

Fig. 1 (continued) Application of the gradient program and UV detection at 231 nm (B). fluorescence detection: $\lambda_{ex}/\lambda_{em}$=231/470 nm - (C); $\lambda_{ex}/\lambda_{em}$=336/425 nm – (D) for the standard of OPA amino acid derivatives: 1 - aspartic acid (K-asp); 2 – glutamic acid (K-glu); 3 – aspargine (Asp); 4 – glutamine (Glu); 5 – histidine (His); 6 – serine (Ser); 7 – arginine (Arg); 8 – glycine (Gli); 9 – threonine (Tre); 10 – tyrosine (Tyr); 11 – alanine (Ala); 12 - taurine; 13 – methionine; 14 – valine (Val); 15 – Se-Met; 16 – phenylalanine (P-ala); 17 – 2,6-diaminopime-lic acid (DAPA); 18 – *iso*-leucyne (*i*-leu); 19 – leucine (Leu); 20 – Se-Cys; 21 - Cys; 22 – homo-cystine; 23 – lysine (the procedure: 30µl AA standard, 0.5ml OPA and 15 µl 1 M NaOH)

As can be observed from obtained results (Table 2 and Fig. 1 B) Se-Cys, cystine (Cys) and h-Cys could be satisfactory quantified using UV detection at 231 nm in comparison with fluorescence detections modes (FD-1 and FD-2) and the typical UV detection at 336 nm. The poor detectable property of these OPA-amino acids is agreement with our previous studies. These OPA-amino acids possess very poor fluorescence response due to the presence of S-S and particularly Se-Se bonds in molecules of these amino acids. On the other hand, only one S or Se atom in a molecule of Met and Se-Met as well as longer length of the C-chain than in Cys or Se-Cys compounds resulted in lower suppression of fluorescence response of OPA-Met and OPA-Se-Met. The value of the UV/FD-2 ratio of OPA-h-Cys is smaller than the UV/FD-2 ratio of OPA-Cys (Table 2) due to the longer distance between $-NH_2$ group and S atom in a molecule of h-Cys. Moreover, the presence of Se-Se bond in Se-Cys most efficiently stimulate decomposition of the chromophoric group of OPA-Se-Cys, therefore, the value of R_{336nm} of this OPA-Se-Cys is highest (Table 2).

The yield of the derivatizing reaction and stability of OPA-AA derivatives was examined at -29°C with respect to storage time of processed AAs in standards and bovine blood serum. Obtained results documented that OPA-AAs with the exception of derivatives of Cys, Se-Cys and h-Cys were stable, when processed AA standards were protected from the light and stored for 24 h at -29°C. Interestingly, a small increase of the peak area of was observed after 24 h. So we suggest that the OPA derivation reaction reach an equilibrate state after 80 min at –29°C. On the other hand, only a minute decrease (7-20%) of the concentration of OPA-glycine derivative in processed AA standards and rats' and ovine' serum samples was observed after 24 h storage at -29°C, whereas the substantial decomposition of Se-Cys, Cys and h-Cys derivatives were found in the same conditions in processed AA standards (Table 2) and bovine blood serum samples. Interestingly, contrary to observed stability of OPA-AA derivatives in processed AA standards, the small decrease (<12%) of the concentration of AA adducts in processed rats' and ovine' serum samples was found after 24 h storage at -29°C. The possible explanation is that the higher content of water solution (130 μl) in processed serum samples than in processed AA standards (45 μl) stimulated decomposition of OPA-AA derivatives.

Conclusion

The chromatographic procedures for the assays of free and protein AAs described show the advantages of the *pre*-column derivatization method. Elimination of the *post*-column reactors yield a less expensive and time consuming, and a more versatile analytical tool. Accurate and rapid analysis of AAs and especially more stable of OPA-AA derivatives was achieved. Our new chromatographic method is most suitable for simultaneous determination of Se-Cys, Se-Met, h-Cys or/and DAPA (traces of bacteria protein) together with other free or protein AAs. The minimum detectability for AAs could be improved by exciting the OPA-AAs derivatives at 336 nm or the UV detection at 231 nm and by applying more concentrate processed samples (two-threefold). A long and high resolution C_{18}-column enabled satisfactory routine separation and quantify of all OPA-AAs from endogenous species present in liver, muscles and blood serum samples.

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APPLICATION OF SEC AND RP-HPLC TO THE INVESTIGATION MECHANISM OF HYDROGERMYLATION REACTION PRODUCING TRIBENZYLGERMANYL DERIVATIVES

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Abstract

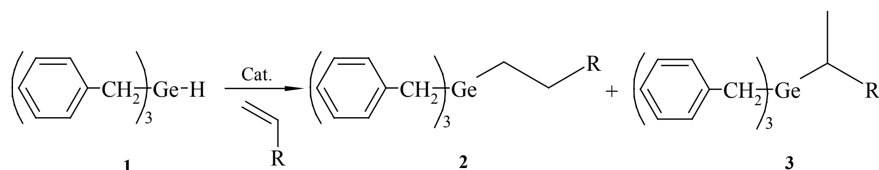
Although germane-organic compounds are present in many biological cells they role is clear for now. From the other side some of them are intensively investigated because of their predicted anti-carcinogenic properties. In the paper synthesis of tribenzylgermane compounds is described. The mechanism of these reactions has been performed using NMR as well as size exclusion chromatography and reversed-phase HPLC.

Introduction

The hydrogermylation reaction is the one of the most convenient way to the direct process in producing compounds with germanium carbon bonds. Therefore efficiency and directs of reactions have been insensitively investigated [1].

Synthesis

Asymmetric tribenzylgermane compounds [2-4] of general formula $(C_6H_5CH_2)_3GeR$, **2** or **3** have been synthesized by the reaction of non-saturated compounds, as it is presented on Scheme 1.



R = $-CH_2C{\equiv}N$; $-CH(OEt)_2$

Scheme 1.

It was observed that, depending on the reaction conditions, hydrogermylation may proceed both on double and triple bonds. The reaction directions and yields depend on its conditions (temperature, time, concentrations, presence of some compounds, like allylcyanide, *etc*.) and used catalyst ($H_2PtCl_6{\cdot}6H_2O$ or PtO_2). It turned out that the hydrogermylation of triple bonds offers a greater synthetic challenge than double bonds because of their higher reactivity and broader product distribution. Good yield and stereo-selectivity were maintained by control a wide range of substrates. The major by-product of these reactions is γ-isomers (Table I).

The reaction has been performed by mixing of equimolar amounts of germane and olefin with 100 ppm (i.e. 100 μmol per mole of alkene) of catalyst, in a sealed tube, under

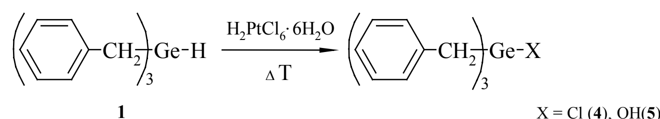
argon. The crude products are then filtered through β-alumine (aluminum oxide) using diethyl ether, concentrated in vacuum and finally applied to a silica gel column (as a mobile phase pet. ether – diethyl ether, 2:1 vol/vol was used). As a result 75 – 95 % compounds are obtained as colorless oil.

Table I.

Hydrogermylation Conversion with
Tribenzylgermane on Functionalized Olefins $H_2C{=}CH_2\text{-}R$

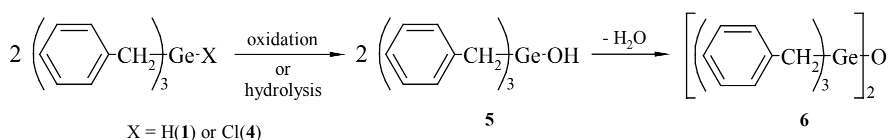
Entry	Olefins R - Functional group	Reaction Conditions							
		temp 60 - 80 °C / 70 h				temp 120 - 140 °C / 32 h			
		$H_2PtCl_6 \cdot 6H_2O$		PtO_2		$H_2PtCl_6 \cdot 6H_2O$		PtO_2	
		Yield * (%)	Ratio[a]*	Yield * (%)	Ratio[a]*	Yield * (%)	Ratio[a]*	Yield * (%)	Ratio[a]*
1	$-CH_2C{\equiv}N$	85	70/10	> 95	90/10	75-95	85/10	95	80/20
2	$-CH(OCH_2CH_3)_2$			> 95	100/-	85	90/10		

* Calculated on the basis of ^1H NMR spectrum and GC/MS.
[a] (γ / β) adducts.



Scheme 2.

It is worth to note that the addition reaction to triple nitrile bonds (-C≡N) is not observed under usual reaction conditions (70 h, 60 – 80ºC), even after heating. Replacement of platinium dioxide by chloroplatinic acid (H_2PtCl_6 x 6 H_2O) decreases yield of reactions of the nitrile derivatives (4-tribenzylgermanyl-butyronitrile and 3-tribenzylgermanyl-butyronitrile), as it is presented in Table I and on Scheme 2. Similar results are obtained by the hydrolysis of tribenzylchlorogermane (**4**) or oxidation of hydrogermane (**1**) (Scheme 2 and 3). Obtained organogermanol (**5**) can be isolated by crystallization. Its spontaneous dehydration leads directly to hexabenzyldigermoxane (**6**) (<2% of all product) (Scheme 3).



Scheme 3.

Chromatography

Size exclusion chromatography and reversed phase HPLC with diode array spectrophotometric and spectrofluorometric detectors have been used to study these mechanisms. Because of the similarity of investigated compounds we have assumed that they are characterized by the same extinction coefficients. Therefore chromatographic peaks surface areas were proportional to their concentrations and, indirectly, to yields of adequate reactions. Peaks at two characteristic wavelengths were observed: 240 nm – characteristic for aromatic groups around germane and 265 nm – characteristic for nitrile groups.

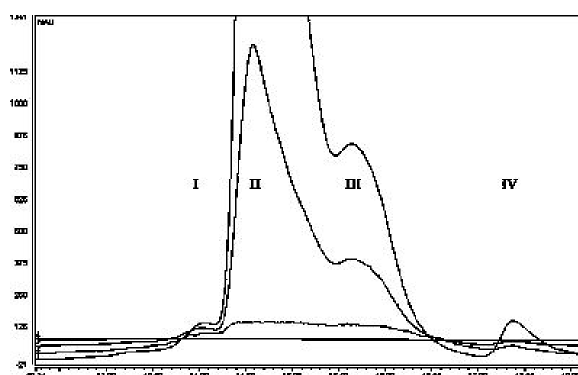


Fig. 1. SEC chromatogram of the products of reaction. Chromatographic conditions: Plgel column - 5 μm, 50 Å, 600x7.8 mm I.D.; mobile phase – dichloromethane, UV detector – 210, 254, 280 and 320 nm; temperature - ambient, flow rate – 1 ml/min., injection volume – 40 μl.

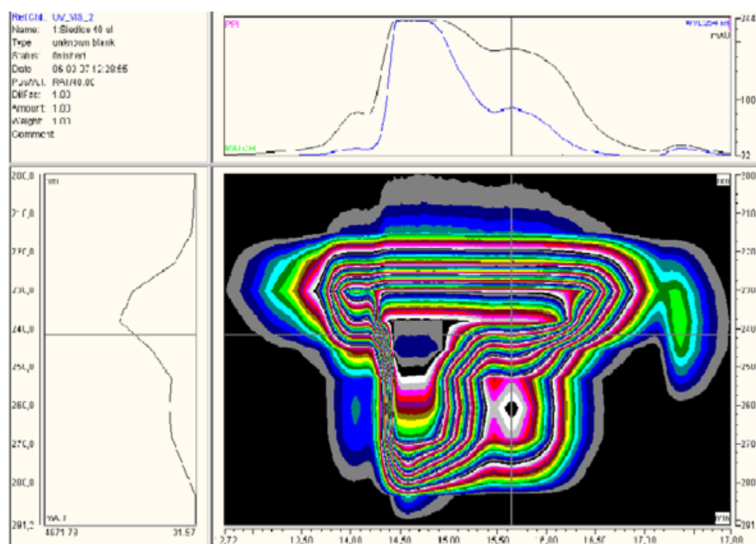


Fig. 2. Three dimensional SEC chromatogram of the products of reaction. Chromatographic conditions as on Fig. 1.

It turned out that at 240 nm relative peaks heights I:IV:III:II were equal 0.1:1:10:100 (Fig. 1). Compounds are eluted inversely to their molecular weights. However, only two peaks (4-tribenzylgermanyl-butyronitrile and 3-tribenzylgermanyl-butyronitrile) were observed at 265 nm (left part of Fig 2). It was found good correlation between these peaks areas and predicted reaction pathways, confirmed by NMR spectra. Additionally, side reaction have been studied at 240 nm (characteristic wavelength for tribenzylgermane group). Peak I belongs to compound containing hexabenzyldigermoxane (**6**) in the molecule.

Similar results were obtained using reversed phase HPLC. It turned out that obtained compounds are characterized by strong fluorescence with maximum at 232/240 nm (Fig. 3). These wavelengths were used for the recording chromatograms.

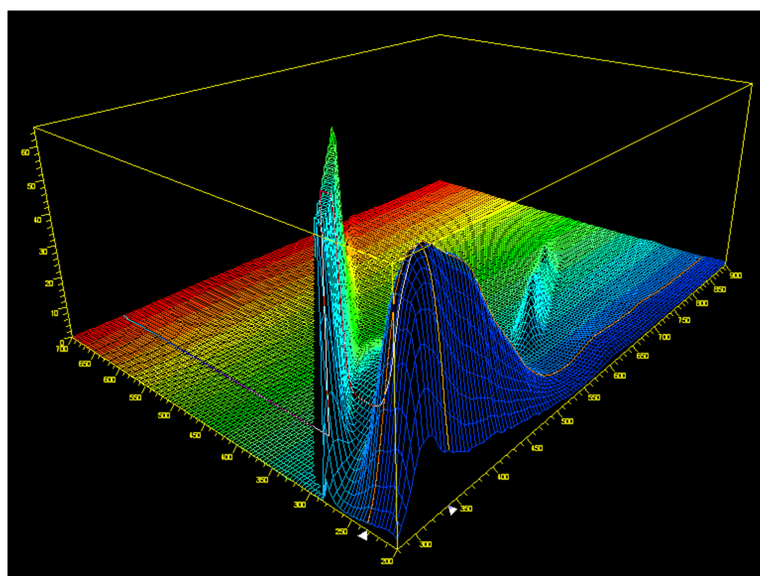


Fig. 3. The stop-flow three dimension (signal vs. excitation and emission wavelengths) fluorescence spectra of 4-tribenzylgermanyl-butyronitrile.

Conclusions

It turned out that HPLC can be used to the study reaction mechanism of synthesis of tribenzylgermane.

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OPTIMIZATION OF CONDITIONS FOR ISOLATION AND DETERMINATION OF BISPHENOL A IN MILK

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Key words: bisphenol A (BPA), powder milk, silica modified, gas chromatography

Abstract

This work describes some results of identification and determination of bisphenol A (BPA) in the powdered milk, by applying the gas chromatography. To determine BPA contents in the milk, and to reduce the matrix interference associated with the constituents of the powdered milk, we performed the following activities. First, we ultra-centrifuged the dissolved milk solutions. Second, we pre-concentrated the analytes in the supernatant using a C_{18} solid-phase extraction column. Finally, we used gas chromatography for the determination of BPA in the samples under study. A recovery of bisphenol A introduced into the milk samples was also performed, with recovery result located at 91%÷94% .

Introduction

Some recent research showed an influence of many chemical substances, so far treated as of no importance, to the health of the humans and the animals. Such chemicals as natural and synthetic hormones, metalorganic compounds, durable organic compounds, monomers, and some chemical additions used in the plastic industry, are able to disturb the natural hormonal balance of the human body, as well as to cause several diseases both for the humans and the animals [1-3].

Particular attention has been paid recently to Bisphenol A (BPA) [4-7]. This compound is used for many industry activities, including the synthesis of polycarbonate plastics, epoxy resins, and polyacrylates. These plastic materials are massively used for the productions of (among others) baby bottles and internal coating layers for the packings for baby food industry, such as powdered milk and milk mixtures [7].

Recently, many publications confirmed estrogenic activities of BPA *in vivo*, even taken in very little doses. These are foetus and little babies that are the most sensitive group taking into account hormonal aspects of BPA exposal. It seems that the main way of BPA penetration is related with milk, both breast milk and some diet supplements (e.g., powdered milk).

This work is concentrated on determination of BPA in the milk and milk-derivative products. The main goal of this work was to propose optimum conditions for the isolation and further determination of BPA in the milk samples, by the use of SPE, gas chromatography coupled with Flame Ionization Detection (FID), and Low Resolution Mass Spectrometry (LRMS).

To determine BPA contents in the milk, and to reduce the matrix interference associated with the constituents of the powdered milk, we performed the following activities. First, we ultra-centrifuged the dissolved milk solutions. Second, we pre-concentrated the analytes in the supernatant using a C_{18} solid-phase extraction column, and new sorbent with chemically bonded ketoimine groups. Finally, we used gas chromatography for the determination of BPA in the samples under study. To this goal, we applied gas chromatography with flame ionization detection (FID) and low resolution mass spectrometry (LRMS).

Apparatus and Reagents

The chromatographic separation was performed using the following hardware.

- Gas chromatography VARIAN CP-3380 equipped with flame ionization detector (FID). A CP-SIL 5 CB (30 m x 0,32mm; DF=1,0) capillary column was used. The analysis was performed in the temperature of 250ºC. Helium was the carrier gas. All the work was carried out in a constant flow mode set at 3.5 ml min^{-1}.
- Gas chromatography PERKIN ELMER AUTOSYSTEM XL TURBO MASS. Equipped with low resolution mass spectrometry detector (LRMS).A DB-5 (30 m x 0,25 mm x 0,25µm) capillary column was used. Temperature programme: 2 min at 150ºC, then programmed at 30ºC min^{-1} to 270ºC and held for 10 min; injector temperature: 250ºC; detector temperature: 300ºC.

A Bakerbond spe vacuum manifold was used for the elution of SPE columns.

Analyzed compound investigated in the separations were purchased from Sigma – Aldrich, and had a purity of equal or greater than 98%. The water was purified in the Milli-Q apparatus (Millipore S.A. 67120 Molsheim, France). The structure of the bisphenol-A is shown in Fig. 1.

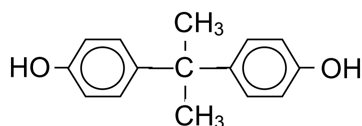


Figure 1. The structure of 2,2-bis-(4-hydroxyphenyl)-propane (BPA)

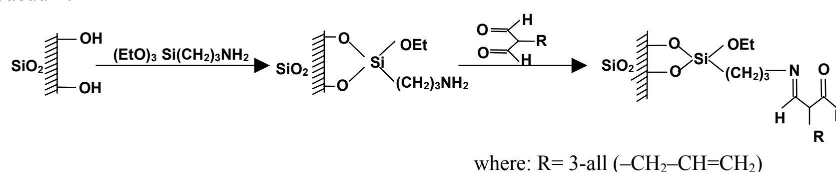
All standard stock solutions were prepared in methanol and used after proper dilution with the same solvent. Silica gel (Baker Analyzed^{R}) was purchased from J.T.Baker.

Sorbent preparation

5 g of dry silica was immersed in a mixture of anhydrous xylene and 3-aminopropyltriethoxysilane. The mixture was boiled for 12 h in a vessel equipped with a reflux condenser. The contents were continuously stirred and carefully protected against the moisture. Unreacted silane was extracted with xylene and hexane in a Soxhlet apparatus. After that it was dried under vacuum and finally subjected to the so-called „end capping" reaction with hexamethyldisilazane in order to deactivate free silanol groups remaining at its surface.

The second step was bonding of amino groups using an appropriate derivative of 3-pentano-2,4-dione. As previously, reaction was performed under continuous stirring in anhydrous xylene

and lasted 12 h. The system was protected against the moisture. The final product was extracted subsequently with xylene and hexane in a Soxhlet apparatus. Finally, silica was dried under vacuum.



where: R= 3-all ($–CH_2–CH=CH_2$)

Figure 2. A scheme of the modification procedure for the packings.

Sample preparation

The powdered milk were purchased from polish supermarkets. Accurately weighted portion of 0.5 g of the analyzed milk was dissolved in 5 ml 50% (v/v) ethanolic solution. The sample solution was mixed for 2 min in an ultrasonic chamber. Such prepared sample was centrifuged for 40 mins at 5000 rpm, and finally filtered through a membrane filter 3W.

Before extraction, each SPE cartridge was conditioned with 5 ml of methanol, and 15 ml of deionized water on an SPE manifold. Such prepared columns were injected by certain amount of the milk samples. Once the total amount of a sample was put, the sorbent was dried for 10 minutes under vacuum, and the preconcentrated compounds were washed away by the use of 3 ml of methanol. The extract was dried up and further dissolved in 0.25 ml of methanol. The obtained samples were measured by GC/FID and GC/MS systems. The MS detector was set up to monitor the ion 228, 213, that is typical of BPA.

Results and discussion

Calibration graph

Dependencies between the peak area and the compound concentration were determined as a result of a chromatographic analysis of samples with added BPA. Based on these dependencies, the calibration graphs were prepared. To this goal, some pattern solutions were used with the BPA concentration ranging from 2 to 50 μg cm^{-3}. The final peak area was taken as an average of three experiments in turn. Calibration plot was described with the general equation: $y = ax + b$, where y is the peak area, and x – the amount of determination compound in μg mL^{-1}. For the observed range of concentration values, a linear route is observed of the calibration curves for calibration coefficients greater than 0.9997. The obtained calibration curves are characterized by very good linearity.

Recovery tests were performed for the powdered milk, with significant, known amount of BPA added (0.5 μg g^{-1}). These tests were performed using the above described method, with the recovery result obtained for C_{18} sorbent equal to 91% and for sorbent with chemically bonded ketoimino groups – 94 %.

Determination of BPA in powdered milk

The powdered milk for the analysis of BPA concentration was purchased from polish

supermarkets. The milk was produced by two independent companies. BPA determination was performed according to the procedure described in the Experimental section. The obtained samples were measured by GC/FID and GC/MS systems. Sample chromatograms of the samples under study are presented in Figure 2 (GC/MS) and 3 (GC/FID).

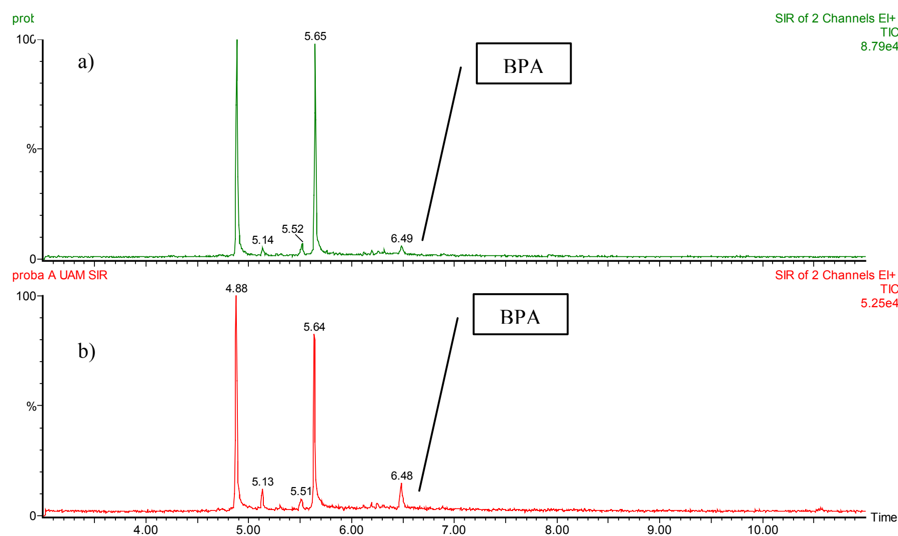


Fig.2. Sample GC/MS chromatograms of powdered milk: (a) pure powdered milk, (b) samples with known additions of BPA

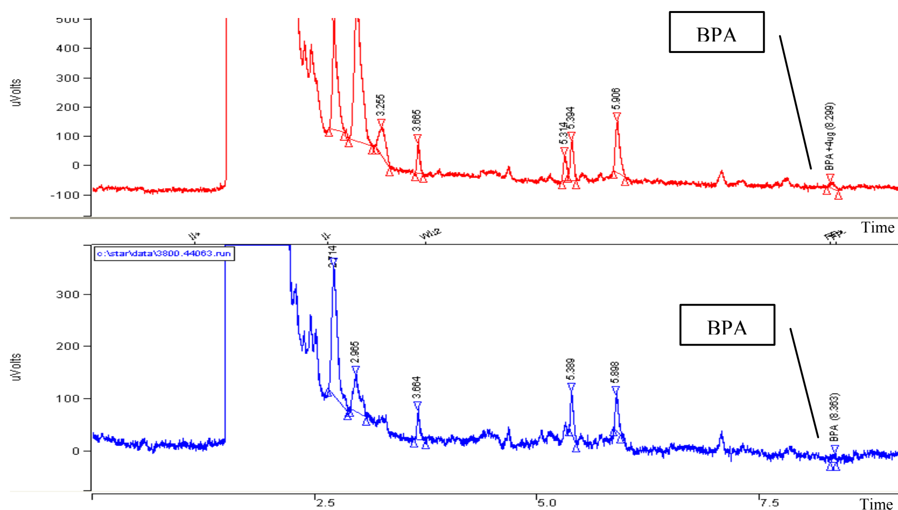
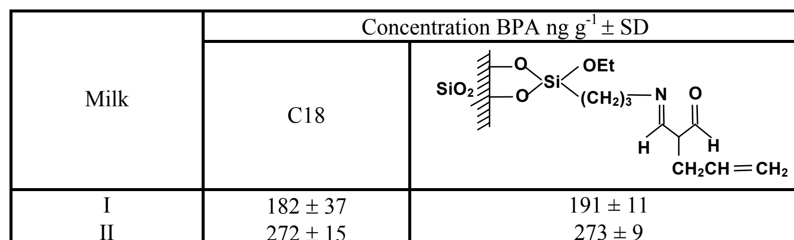


Fig.3. Sample GC/FID chromatograms of powdered milk: (a) pure powdered milk, (b) samples with known additions of BPA The results of BPA determination (GC/FID) in the powdered milk are presented in Table 1.

Table 1. BPA determination in the powdered milk samples

Milk	Concentration BPA ng g^{-1} ± SD	
	C18	
I	182 ± 37	191 ± 11
II	272 ± 15	273 ± 9

Conclusions

The proposed method of determination of BPA in powdered milk is sensitive and selective. A combination of the solid phase extraction SPE and gas chromatography GC permits the determination of this compound at a level of ng g^{-1}. The proposed method of extraction and preconcentration of the analyte is characterized by high amount of recovery 91%÷94%. As proven by our experiments, newly synthesized sorbent with chemically bonded ketoimine groups may be used for preconcentration of BPA by the use of the SPE method.

The measured concentration of BPA in the powdered milk belongs to the range 182 to 273 ng g^{-1}. The experiment proved that BPA is present in the powdered milk, that is particularly dangerous for the babies.

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MICELLAR LIQUID CHROMATOGRAPHY IN ANALYSIS OF BISPHENOL A

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Key words: Bisphenol A, Water analysis, SPE, Micellar Liquid Chromatography

Introduction

There are a number of chemical substances that disturb regular performance of the hormonal system. They are referred to as endocrine disrupters [1, 2] and their undesirable effect is felt by both men and women. The substances disturb the hormonal equilibrium of organisms, which is particularly dangerous in developmental age, when the changes can be irreversible. Destabilisation of the hormonal system can lead to a number of physiological effects, e.g. incorrect functioning of the thyroid gland hormones in the intrauterine life can cause a permanent brain damage. Endocrine disrupting compounds (EDCs) can be responsible for neurological conditions, problems with reproduction and development, disturbances in the immunological system and neoplasmic changes. The mechanisms of their activity in living organisms are different but the most often met are those based on imitation of the activity of natural hormones, acting as their antagonists, blocking of receptors, entering into reactions with the hormones or modification of the synthesis of hormones [3, 4].

The endocrine disrupting compounds EDCs are divided into three groups: pharmaceutical e.g. contraceptive drugs and some therapeutic drugs, natural (estrogens found in plants) and some environmental pollutants. The compounds present in the natural environment and showing estrogenic properties are e.g. organochlorine pesticides, alkylphenols, phthalates, polychlorinated biphenyls and dioxins, organic tin compounds and bisphenol A [5].

Bisphenol A (4,4'-isopropylidene diphenol, BPA) has been used as a raw substance for production of e.g. epoxy resin, polycarbonate, polyester and polyacrylate plastics. It has been used as a fungicide, antioxidant, an agent suppressing inflammability in rubber industry, and plastic production, as a stabiliser in production of polyvinyl chloride. BPA is obtained in the reaction of condensation of phenol with acetone against ion-exchange resin as a catalyst. BPA and its derivatives are potentially hazardous to the consumer health thus their presence and concentration level in food products should be monitored [6, 7].

Polycarbonate plastics (PC) are commonly used for production of food product packages, bottles for water, bottles for infant food, kitchen utensils and elements of medical apparatuses. The substances able to release BPA are used for coverage of inner surfaces of tins for food products or in dental fillings. BPA residues have been found in water and other food products stored in PC packages. BPA is able to get released from the PC package and migrate into the food stored inside. Such a migration is favoured by acidity of the product stored, elevated temperature, mechanical cleaning and the use of detergents for cleaning bottles or other PC packages [6-8].

BPA was also recently re-evaluated by the Scientific Committee on Food (SCF), resulting in the establishment of a provisional tolerable daily intake (TDI) for BPA at 0.01 mg/kg body weight /day [9].

The analysis of BPA has been accomplished by chromatographic techniques, such as HPLC equipped with fluorescence [8, 10, 11], ultraviolet [7, 12, 13] electrochemical [14, 15] or mass spectrometry detection [16-18], gas chromatography [18-22], as well as micellar electrokinetic chromatography [23, 24].

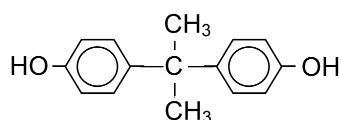
The aim of the study was to propose a fast and simple method of determination of BPA and its derivatives in water being in contact with packages made of BPA containing plastic. The material studied was bottled drinking water distributed through large networks in Poland. Analysis was performed with the preliminary isolation and concentration of the EDCs to be determined by the solid phase extraction (SPE) and followed by liquid chromatography on a chromatograph equipped with a UV detector.

Experimental

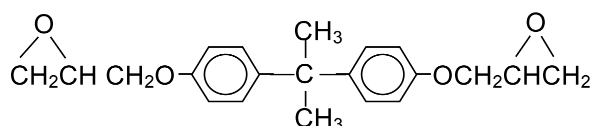
Chemicals and reagents

Methanol (MeOH) was gradient grade for chromatography, 2-propanol and dichloromethane HPLC grade (Merck, Germany), dodecyl sulfate sodium (Aldrich), acetic acid was analytical grade (POCh, Poland).

Standards of bisphenol A (BPA) and 2,2-bis-(4-hydroxyphenyl)-propane-bis-(2,3-epoxypropyl) ether (BADGE), purchased from Aldrich (USA) were used for preparation of individual stock solution in methanol at the level 1 mg/ml. Standard mixtures were made up in MeOH by dilution of stock solutions.



BPA 2,2-bis-(4-hydroxyphenyl)-propane



BADGE 2,2-bis-(4-hydroxyphenyl)-propane-bis-(2,3-epoxypropyl) ether

Water was distilled and then purified by a Milli-Q water purification system (Millipore). Mineral water contained in 18.9 l polycarbonate plastic bottles made by three different producers was purchased at a city market.

Sample preparation procedures

The solid phase extraction was performed with SPE-12G vacuum system (Baker SPE, Germany). Cartridges with sorbent C-18 were placed on a vacuum manifold, conditioned with 5 ml of MeOH /dichloromethane = 1/1, 5 ml of MeOH and 10 ml of deionized water.

For the studies of recovery and analytical precision, sample volumes of 500 ml of tap deionized water were chosen and spiked at the EDCs investigated. After preconcentration, the sorbent was dried in a vacuum system for 20 min. The compounds retained were eluted with 2 ml of MeOH. The eluent was then evaporated to dryness and then reconstituted 250 µl MeOH. The extracts were then injected onto the HPLC system.

Drinking water was analysed by the following procedure: 500 ml of water were extracted by SPE column. The samples were percolated through bonded phase silica extraction tube at a flow rate of approximately 1 ml/min, previously conditioned.

Liquid chromatography analysis

The study was performed using a Hewlett-Packard HP 1050 chromatograph equipped with a UV detector (190 - 600 nm) and a Rheodyne model 7125 injector with a 20 µl sample loop. The chromatographic separations were performed on a column Nucleosil C-18AB (5 µm, 150 x 4.6 mm) (Alltech Associates, Inc.) at 40 ^{o}C. The flow rate of the mobile phase was 1 ml/min. The detection of BPA was performed at 260 nm. The mobile phase was a water solution of the anionic surfactant of sodium dodecylsulfate (SDS, Aldrich. The micellar mobile phase was filtered in vacuum through 0.45 µm filters of cellulose acetate and degassed with helium. The standard solutions of the BPA and BADGE (Aldrich), of the concentration of 1.0 mg/ml were prepared in methanol. The standard solutions of each of the compounds of the concentrations of 10 µg in 1 ml were made in the mobile phase. The water used for preparations of the solutions and the mobile phase was deionised in Milli-Q (Millipore, USA).

Results and discussion

Chromatographic optimisation

Optimisation was indispensable to provide complete separation of the compound studied and to eliminate a possible matrix interference's in the shortest time. For analysis a good separation of the compounds applied was obtained with a micellar mobile phase. The figure presents the curves illustrating the correlation between the retention coefficient and the SDS concentration and the % content of 2-propanol as an organic modifier in the mobile phase.

Determination of BPA and BADGE was performed with the use of a micellar mobile phase composed of 0.2 M SDS and 12% 2-propanol, at the flow rate of 1 ml/min and the separation temperature of 40 ^{o}C.

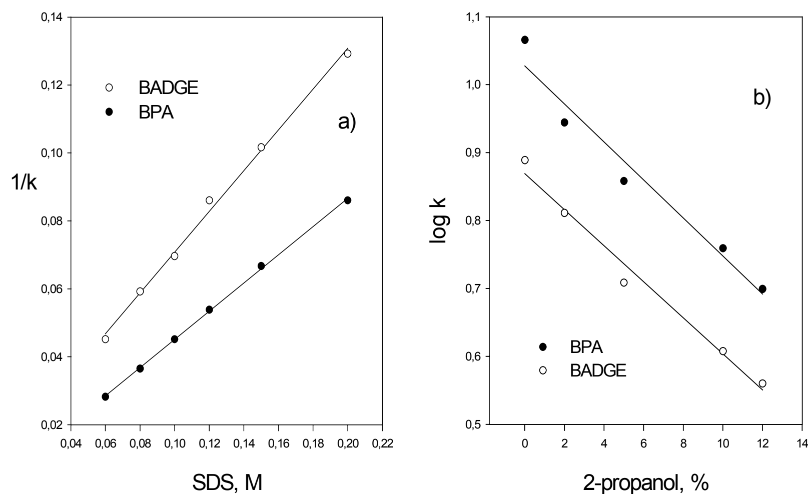


Fig. 1. The effect of a) the SDS concentration and b) the volume fraction of 2-propanol (0.10 M SDS) on the retention behaviour of BPA and BADGE.

Calibration. Limit of detection. Recovery

Calibration was performed for a mixture of standard solutions of the compounds analysed. To determine the linear range of the response to direct injection, 20 µl of a standard solution in deionised water were injected. The calibration curves were constructed using the eight concentration levels and each concentration was injected in triplicate. The calibration plots were approximated by the equation y = ax + b, where y – is the peak area, and x – the concentration of determination compounds in mg/L. Good correlation coefficients, higher than 0.997, were obtained. The detection limits (LOD), obtained from the peak height of the compounds analysed, were defined as the concentration of 3 SD of the baseline signal. The parameters of the resulting calibration lines and the limit of detection are listed in Table 1.

Table 1. Parameters of the calibration curves and the detection limits for the compounds analysed

	a	b	r^2	Range [µg/L]	LOD [µg/L]
BPA BADGE	36.4 30.4	-2.2 74.0	0.9999 0.9976	0.50 – 100 1.0 - 100	0.30 0.60

Table 2 presents the values of recovery and relative standard deviation of the EDCs determined in water. The compounds analysed were introduced into water in concentrations 1 µg/L.

Table 2.Recovery of analyzed EDCs extracted from 500 ml spiked tap water (1 μg/L) after SPE on C-18. (n = 6)

	Recovery [%]	R.S.D. [%]
BPA	93.3	3.86
BADGE	85.4	4.23

Determination of BPA of water

Three types of mineral water samples contained in polycarbonate plastic bottles were purchased from a city market. Determination of the concentrated compounds was made using the sorbent C-18. The analysis was performed by the use of the standard addition method. Results are presented in Table 3. Two exemplary chromatograms obtained as a result of the analysis performed are shown in Figure 1. No BADGE compound was detected in the mineral water samples analysed.

Table 3. Results of the drinking water analysis (n = 6)

	BPA [μg/L±SD]	R.S.D. [%]
I	0.50 ± 0.03	4.8
II	0.55 ± 0.08	4.9
III	0.41 ±0.05	3.9

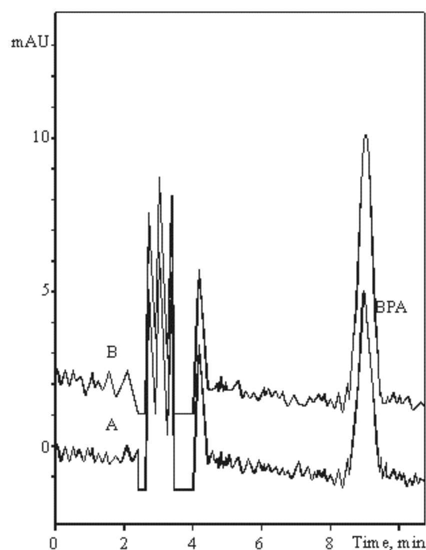


Fig. 1. Exemplary chromatograpms obtained for sample of mineral water (sample 1), A) without artificially added BPA, B) with a certain amount of BPA added.

Conclusions

The method proposed involves concentration of BPA residues in drinking water samples by the solid phase extraction method and their analysis by the micellar liquid chromatography with UV detection. The method is simple, sensitive, fast and characterised by high recovery, e.g. for BPA the recovery was 93.3%. The detection limit is 0,3 μg/L of the water sample studied. The routine use of this method to analyse contaminated water samples depends on improvement of the techniques of sample extraction.

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BIODEGRADATION OF BROWN COALS CAUSED BY FUNGI AND BACTERIA

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Introduction

Biodegradation is one of the most important processes changing composition of bituminous fraction of organic matter and affecting results of geochemical investigations [1]. The process was extensively investigated for crude oils since it strongly affects oil quality leading to formation of naphthenic type petroleum, tar mats in oil deposits or even heavy tars poor in aliphatic components. In the case of brown and bituminous coals only limited number of research was done up to this moment [2]. The effects of the process are generally described as the minor and additional factor influencing coal properties comparing to coal oxidation [3]. However, it was found that biodegradation can significantly alter organic matter of coals, mainly removing their bituminous fraction what changes their chemical and technological properties [4]. The main problem in this research is similarity of results caused by biodegradation and water washing in bituminous fraction of brown coals.

Experimental

To assess the influence of the process in selected brown coals the powdered samples were extracted in DCM to receive bituminous fractions. The extracts were separated into aliphatic, aromatic and polar compounds by preparative layer chromatography (Kiesegel 60, Merck). Plates were developed in *n*-hexane.

Aliphatic and aromatic hydrocarbon fractions were investigated by GC-MS with Agilent Techn. gas chromatograph with HP-5 column (60m x 0.25mm), coated by 0.25 μm stationary phase film was applied. The gas chromatograph was coupled to a mass spectrometer operating in EI mode at 70 eV (full scan, 50 - 650 da). The experimental conditions were as follows: carrier gas - He; temperature program: 50 ^{o}C (isothermal for 2 min), heating rate to 175 ^{o}C at 10 ^{o}C/min, to 225 ^{o}C at 6 ^{o}C/min, and to 300 ^{o}C at 4 ^{o}C/min, final isothermal temperature: 300 ^{o}C was held for 20 min. Data were processed with the Hewlett Packard Chemstation software. All compounds were identified by their mass spectra, comparison of retention times to these of standard compounds and literature data and interpretation of MS fragmentation patterns [5-6].

Results

The following changes were found in chemical composition of brown coals affected by biodegradation caused by bacteria and/or fungi.

- Extractability of the biodegraded samples is lower in all affected brown coal samples independently of biodegrading organisms.

- Biodegradation caused by bacteria leads to gellification of brown coal organic matter during its decomposition in a deposit. In composition of brown coal extracts it is reflected by much higher content of hopanes (or hopenes) and moretanes (pentacyclic triterpanes) comparing to non-affected samples. These compounds all come from bacteriohopanetetrol acting as a rigidifier of bacterial cell walls.

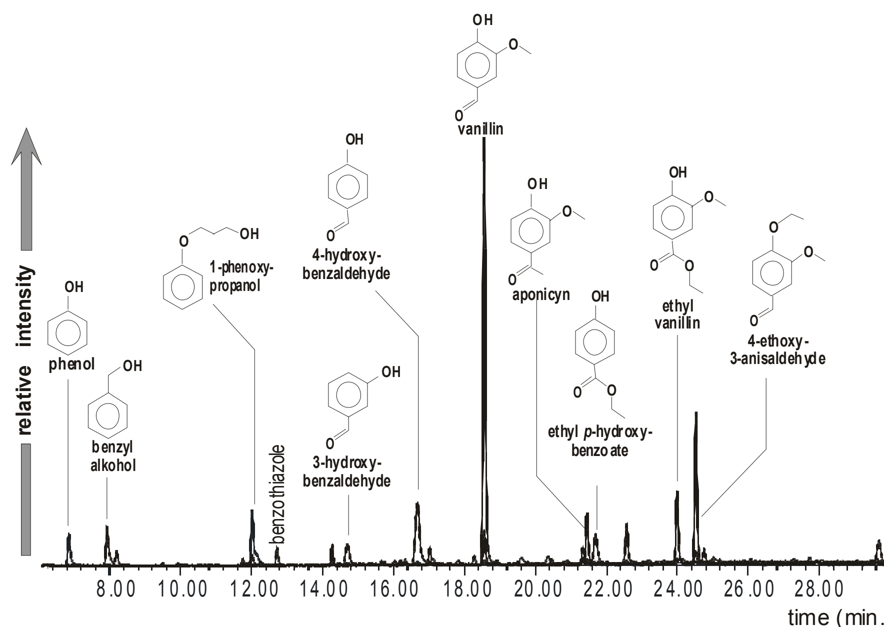

Fig. 1 Distribution of lignin degradation products in brown coals (The Konin Brown Coal Basin) m/z = 94+108+121+151

- Fungi influence is reflected by decomposition of macromolecular fraction of brown coal (Fig. 1). This type of biodegradation can lead to the increase in concentrations of phenolic derivatives such as vaniline, aponicyne, 3-methylacetophenone, acetophenone and ethyl ester of vanilinic acid in the case of the conifer-originated organic matter and 4-hydroxybenzaldehyde, benzylidenacetone, 3-phenoxy-1-propanol and *p*-cresol in the case of grass-originated brown coals.
- Bacterial degradation may be also indicated by presence of noroleananes and norditerpenes (Fig. 2), compounds produced from their geochemical precursors oleanenes and diterpenes.

Norhopanes, often occurring in heavy biodegraded crude oils or tars, were not found in biodegraded brown coals, possibly due to relatively high oxygen content in a depositional environment. The pathway of hopanes biodegradation to norhopanes requires anoxic conditions during the process [6].

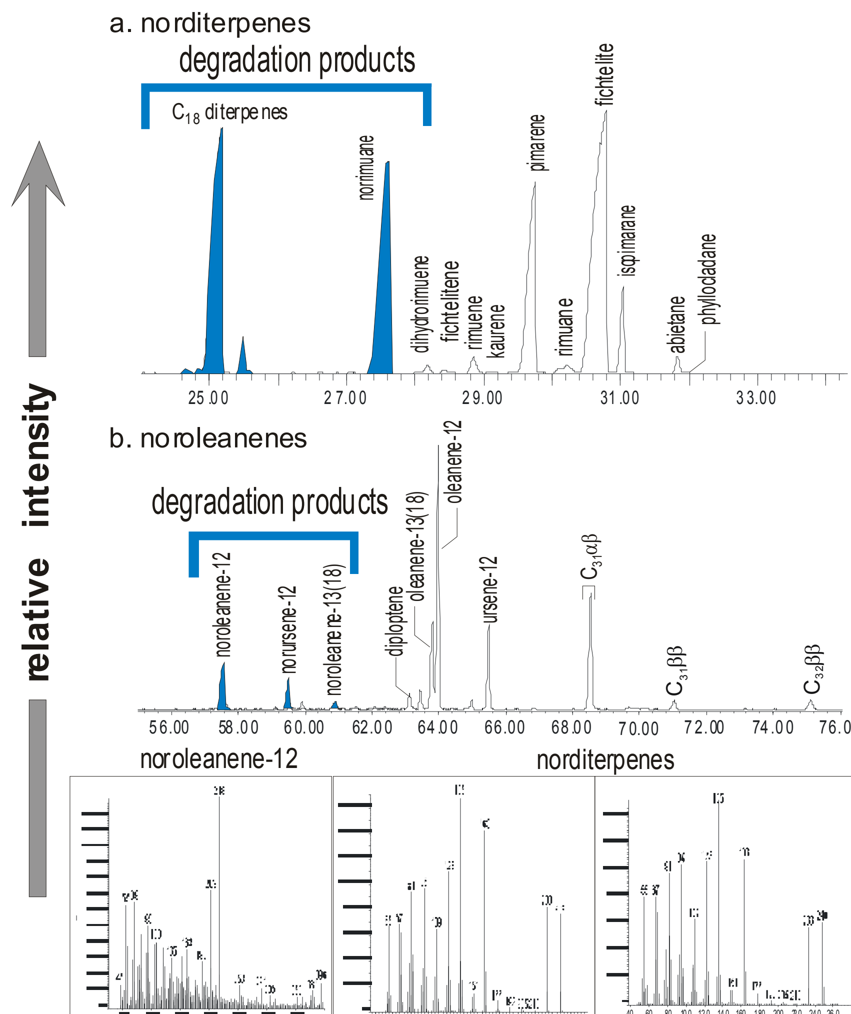

Fig. 2 Compounds formed as a result of biological degradation of organic matter of brown coal (the Turów mine)

Conclusions

It was found that biodegradation of organic matter of the investigated brown coals could be performed both by bacteria or fungi. Both bulk group composition of the extracts and the occurrence of particular compound groups may be affected by the process; however in many cases the changes are similar to these caused by water washing, for example *n*-alkanes removal. Since both water-washing and slight biodegradation can cause the removal of lighter *n*-alkanes it is difficult to use this feature to differentiate biodegraded and water-washed brown coal.

However, it is possible to identify biodegraded brown coals due to the occurrence of characteristic phenolic products being monomers of macromolecule lignin. Their presence in the extracts indicates the possibility of the partial degradation of this resistant component present in sedimentary organic matter. It seems that xylithic brown coals show higher resistance to biodegradation than detritic brown coals, probably due to the diterpenoids presence in wood tissue. These compounds having antifungal and anti-bacterial properties can partially protect lithotypes rich in them. Since detritic brown coals originate from primal organic matter deriving from grasses [7] poor in diterpenoids they are more susceptible to bacterial of fungal attack than xylithic brown coals.

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PLC AND GC-MS IN ASSESSMENT OF CHANGES IN CHEMICAL COMPOSITION OF BROWN COAL EXTRACTS CAUSED BY WATER WASHING

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Introduction

Water washing affects the composition of low-molecular fraction of sedimentary organic matter by removing its lighter and more soluble components [1]. The process concerns especially unconsolidated permeable sediments containing organic matter at relatively early stages of thermal evolution (diagenesis). Such organic material still contains high proportions of heterocyclic compounds or compounds substituted with polar functional groups which are easily dissolved and removed with water. Many factors play role in the process, such as chemical composition related to biogenic origin of organic matter and stage of evolution. Moreover, both the mineral matrix and macromolecular fraction of organic matter can influence water washing by their variable composition. Due to these considerations it is important to find means to assess a range of alterations caused by the process.

Experimental

Brown coals of several brown coal mines were selected for investigations. Powdered samples were extracted with CH_2Cl_2. To estimate group composition of brown coal bitumen the extracts were separated into aliphatic, aromatic and polar compounds by preparative layer chromatography (Kieselgel 60, Merck). *n*-Hexane was used as a developer.

Aliphatic and aromatic hydrocarbon fractions were investigated by GC-MS with Agilent Techn. gas chromatograph equipped with HP-5 column (60m x 0.25mm), coated by 0.25 μm stationary phase film. The gas chromatograph was coupled to a mass spectrometer operating in EI mode at 70 eV (full scan, 50 - 650 da). The experimental conditions were as follows: carrier gas - He; temperature program: 50 ^{o}C (isothermal for 2 min), heating rate to 175 ^{o}C at 10 ^{o}C/min, to 225 ^{o}C at 6 ^{o}C/min, and to 300 ^{o}C at 4 ^{o}C/min, final isothermal temperature: 300 ^{o}C was held for 20 min. Data were processed with the Hewlett Packard Chemstation software. All compounds were identified by their mass spectra, comparison of retention times to these of standard compounds and literature data and interpretation of MS fragmentation patterns [1-3].

Results

Several geochemical parameters were proposed to assess extend of water washing alterations in sedimentary organic material of brown coals.

In a bulk analysis water washing may be indicated by the decrease in extracts yields of brown coals (extractability) up to 0,2-0,4% and decrease in contents of aliphatic and/or aromatic hydrocarbon fraction in the extracts (often lower than 0,7%). Polar compounds generally increase their content in the case of less affected samples since they show higher affinity to macromolecular matrix containing functional groups. In samples of advanced water washing they are also removed, especially lighter compounds.

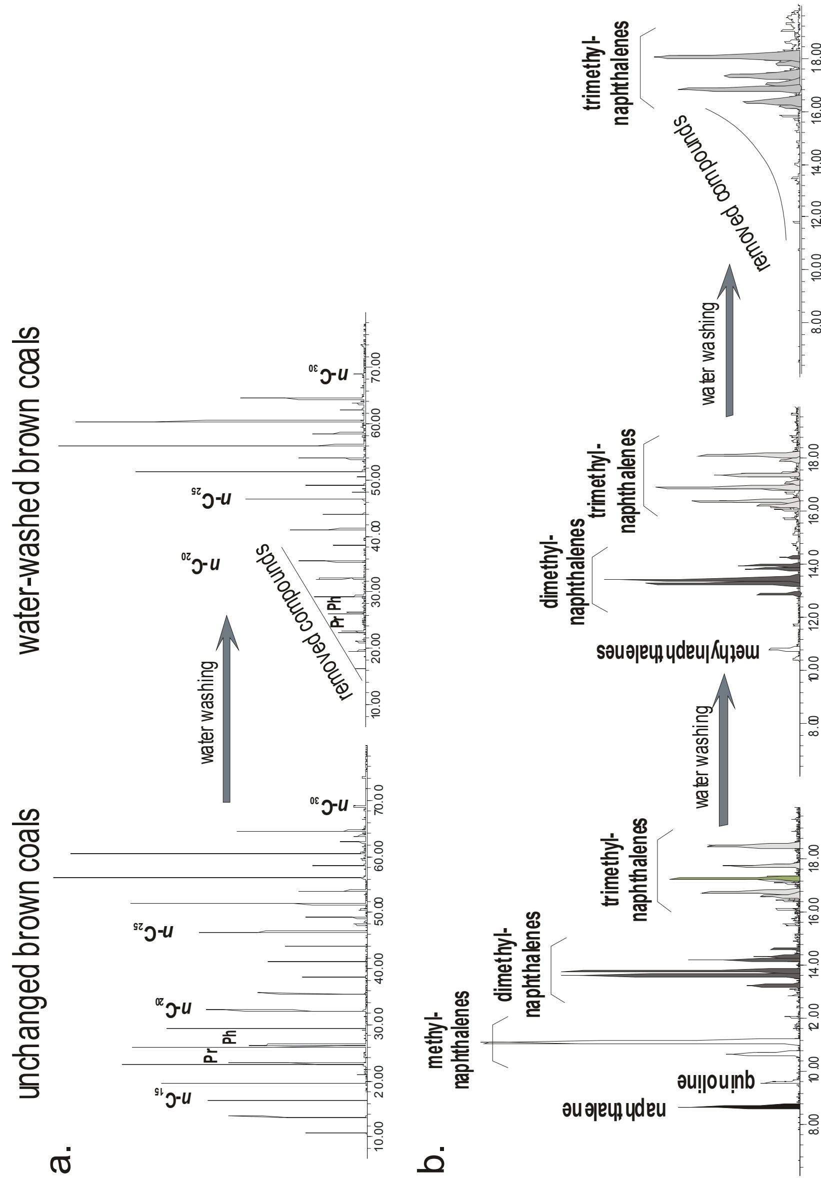

Fig. 1 Changes in distribution of selected compound groups caused by water washing

GC-MS analysis shows the major changes in chemical composition of the extracts such as the progressive removal of lighter *n*-alkanes in the range *n*-C_{12}-*n*-C_{22} together with pristane and phytane, alkylnathphalenes starting from metylnaphthalenes and unsubstituted polycyclic aromatic hydrocarbons with 2-3 aromatic rings (Fig. 1). Biomarker ratios such as 1-methynaphthalene/2-methylnaphthalene ratio, naphthalene/phenanthrene ratio (N/P) and a ration of sums of methylnaphthalenes and dimethynaphthalenes (ΣMN/ΣDMN) were proposed to assess the extent of changes.

Table 1 Proposed orientating scale of water washing degree in the case of organic matter with low thermal maturity

Degree of water washing	Description	Characteristic features in chemical composition
0	no water washing	Distribution of *n*-alkanes unchanged, methylnaphthalenes (MN) with higher concentrations than dimethylnaphthalenes (DMN), isoquinoline, methylquinoline and naphthalene (N) in relatively high contents, light compounds present such as camphor, longipinene, acetophenone and alkylbenzenes up to C_4
1	slightly water washed	Light *n*-alkanes partially removed (a cut distribution envelope up to *n*-C_{15}-*n*-C_{16}), MN with higher concentrations than DMN, removal of lighter compounds with mass weight up to 150, such as acetophenone, camphor
2	slightly water washed	Light *n*-alkanes removed (a cut distribution envelope even up to *n*-C_{20}), MN with concentrations similar to these of DMN, Pr/Ph values decrease, a value of 2-MN/1-MN ratio increases, 8-methylquinoline in low contents, N with content lower than phenanthrene (P)
3	medium water washed	Concentrations of MN lower than these of DMN, N content much lower than P, decreased concentrations of 8-methylquinoline
4	medium water washed	Concentrations of MN much lower than these of DMN, low N and 8-methylquinoline contents, Pr/Ph values approach 0,8-0,9
5	strongly water washed	No N and MN, but 8-methylquinoline may be still present, low DMN concentrations, DNR values decrease significantly, lower MP concentrations, MP and DMP contents similar
6	strongly water washed	No N and MN, low DMN concentrations, low MP and trimethylnaphthalene (TMN) concentrations
7	very strongly water washed	No MN and DMN, low P and MP concentrations, but pyrene (Py), chrysene (Ch) and higher PAHs still occur in extracts
8	very strongly water washed	No MN, DMN and TMN, very low concentrations of P, MP and DMP (or they are absent); Pi and Ch contents decreased, in some cases CPI values are lowered
9	extremely water washed	No P, N, alkylnaphthalenes and alkylophenanthrenes; high weight compounds dominate both in aliphatic and aromatic fractions

Basing on the above findings the 10-point scale of water washing to estimate a range of alterations was proposed (Table 1).

Conclusions

The described here changes are characteristic and their extent can be measured with proposed parameters. Water washing can affect both bulk composition of extracts and biomarker parameters, especially these based on light-weight compounds or functional which are less resistant to dissolving in water. Among them are parameters applying alkylnaphthalenes, mathylphehenthrenes or pristane and phytane. It seems that a special care should be taken interpreting geochemical results concerning organic matter affected by this process.

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GC-MS IN INVESTIGATION OF ORGANIC MATTER PRESENT IN ALLUVIAL AND GLACIAL SEDIMENTS

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Introduction

A number of studies have shown that sedimentary organic matter oxidation by oxygen and nitrate is an important process in controlling groundwater composition since they are the major oxidants dissolved in water [1-3]. The particular importance of redox processes in the buried valley in the Racibórz area has been observed for a long time [4]Kowalczyk, Miotliński, Wojtal, 2002). The elevated concentrations of sulfate and iron suggest that pyrite oxidation is the major mechanism that affects the groundwater chemistry in the area. The existence of dissolved hydrogen sulfide in water and freshly precipitated siderite in the sediment indicates that organic matter oxidation takes place as well.

The overall aim of the study is to evaluate occurrence and composition of organic matter as an important electron donor in the aquifer. Further research will concentrate upon the investigation of the reactivity of organic matter and its contribution in reduction potential within the sediments. This knowledge is required to make a hypothesis related to the processes that do control the water chemistry in the buried valley in the Racibórz area.

Experimental

Organic matter present in fourteen alluvial and fluvoglacial sediments of the Quaternary and Miocene age sampled in two boreholes were the object of geochemical analyses. The host sediments were extracted in a ultrasonic bath with a mixture of DCM:EtOH (4:1; v:v, 3 times) to yield low-molecular organic matter. Since very low extractability, chemical composition of the whole extracts, without group separation was investigated with gas chomatography-mass spectrometry (GC-MS).

An Agilent Techn. gas chromatograph was equipped with HP-5 column (60m x 0.20 mm), coated by 0.25μm stationary phase film. The gas chromatograph was coupled to a mass spectrometer operating in EI mode at 70 eV (full scan, 50 - 650 da). The experimental conditions were as follows: carrier gas - He; temperature program: $50^{o}C$ (isothermal for 2 min), heating rate to $175^{o}C$ at $10^{o}C$/min, to $225^{o}C$ at $6^{o}C$/min, and to $300^{o}C$ at $4^{o}C$/min, final isothermal temperature: $300^{o}C$ was held for 20 min. Data were processed with the Hewlett Packard Chemstation software. All compounds were identified by their mass spectra, comparison of retention times to these of standard compounds and literature data and interpretation of MS fragmentation patterns [5-6].

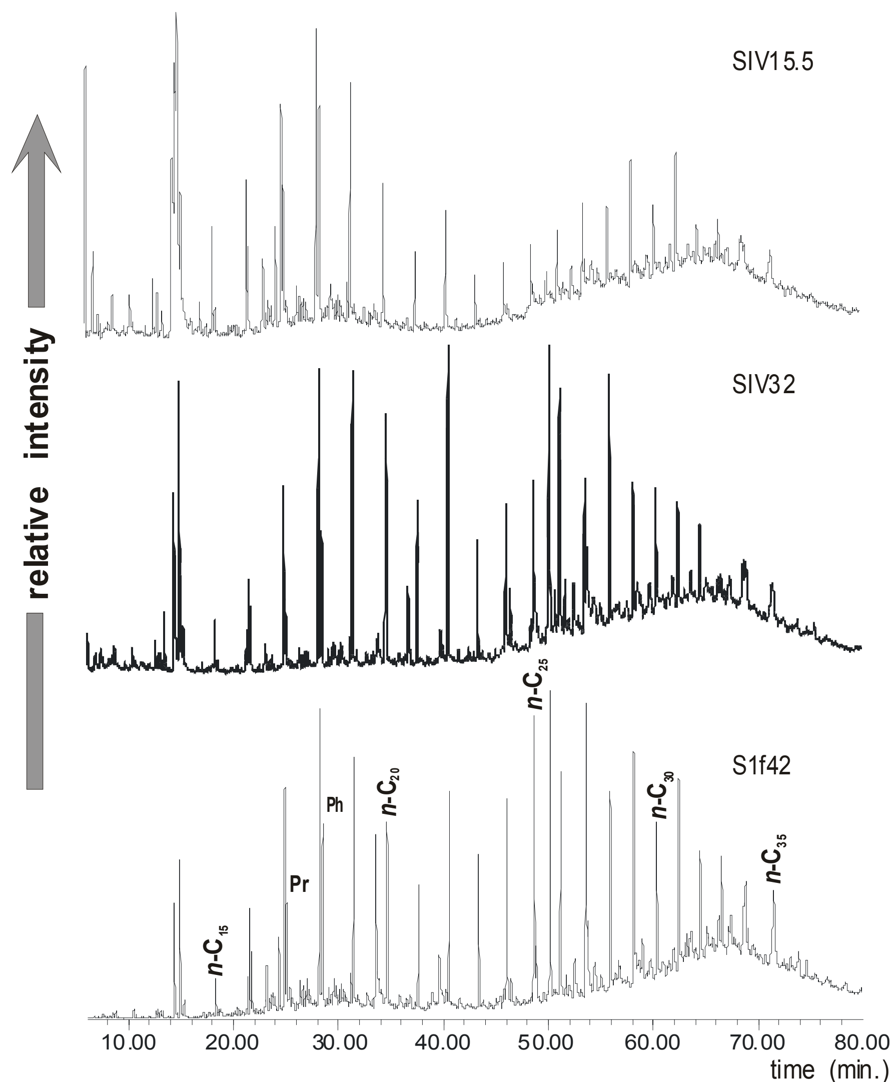

Fig. 1 Three main types of n-alkane distributions in the extracts

Results

The GC-MS results were applied to estimate chemical composition, thermal maturity and possible sources of re-worked and re-deposited sedimentary organic matter using distribution of biomarkers. Extractability is low in the range of 0,010-0,002%. The extracts were dominated by polar compounds, probably from fairly recent material what suggest relatively high reactivity of organic material. Among them were stanols, sterols, phenol derivatives.

carboxylic acids and hydroxyacids. Apart from functionalized compounds the extracts comprised biomarkers indicating organic matter of more advanced evolution such as *n*-alkanes showing three main types of distribution (Fig. 1), acyclic isoprenoids (mainly pristane and phytane), steranes, hopanes and other pentacyclic triterpenoids (friedelin and their derivatives). Only few samples contained diterpanes excluding conifers as a main source of organic matter.

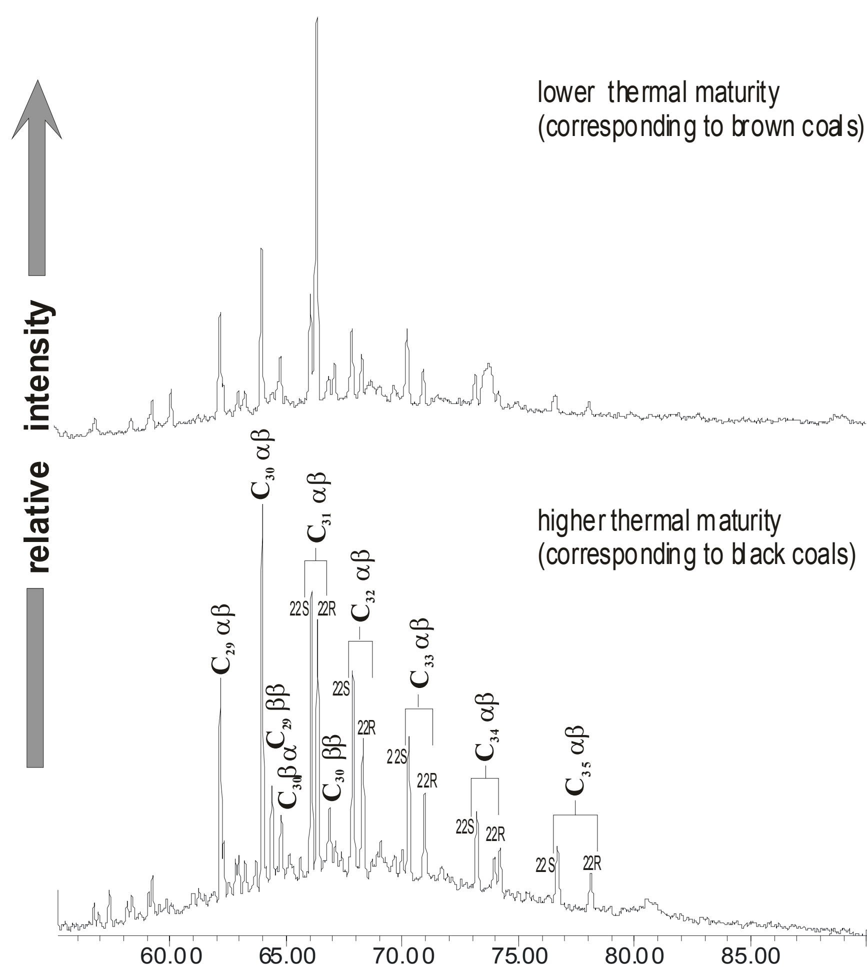

Fig. 2 Distribution of hopanes showing differences in maturity of organic matter

Most groups of compounds, for example hopanes and steranes (Fig. 2) show two different types of distribution related to different thermal maturity. It indicates heterogeneity of geochemical material which was re-deposited from other older source rocks differing in

evolution stage, and probably also in age. However, most of the analyzed material is the mixture of organic matter from these two sources.

Conclusions

It was concluded that there are essentially two sources of organic material present in the investigated sediments.

I. Organic matter of relatively low thermal maturity being at early diagenesis or medium advanceg diagenesis; it may be assumed this organic matter comes from fairly recent biological sources since it contains functionalised polar compounds. Some of these compound may be found in the Tertiary brown coals (vitrinite reflectance ~0,25-0,30%).

II. Organic matter of more advanced maturity being at the stage of moderately advanced catagenesis at least; the values of biomarker parameters and indices testify maturity level corresponding to vitrinite reflectance in the range R_r = ~0,60-75%. Such high maturity indicates organic matter re-deposited as a result of glacial erosion and transportation.

In most cases organic matter seems to be a mixture containing compounds deriving from both these sources; however the Miocene clay contains autochthonous organic matter only. Since polar compounds dominate in the analyzed material present in the extracts it may be assumed that also macromolecular fraction of organic matter is functionalised and shows relatively high reactivity.

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GAS CHROMATOGRAPHY-MASS SPECTROMETRY IN INVESTIGATION OF FIRES IN COAL WASTE DUMPS

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Introduction

Coal mining industry is one of the major waste producers in Poland. Annually, it produces about 0.6-0.7 ton of minestone per ton of exploited coal, collected in 136 waste dumps. These coal wastes, containing usually up to 20% of organic material are susceptible to heating and fire spontaneously occurring in them. Apart from fires and elevated temperature there are also several other factors influencing composition of organic matter wastes such as original heterogeneity of accumulated wastes, their air oxidation and water washing in dumps. Since selfheating and/or spontaneous fire occurring in dumps are potentially hazardous to environment it is important to find what conditions are required for them to occur and what changes in waste organic matter are caused by their various temperature ranges.

Experimental

Twelve samples of coal waste affected by fire to the various extend were collected from the coal waste dump in Piekary Ślaskie (Upper Silesia, Poland) sampling them at various distance (from 0.05 to >4m) from the heat source to show different degree of transformation. Powdered waste samples were extracted with a mixture of DCM:EtOH (4:1, v:v). The composition of total extracts was investigated with GC-MS using an Agilent Techn. gas chromatograph equipped with DB-35 column (60m x 0.25mm), coated by 0.25µm stationary phase film. The gas chromatograph was coupled to a mass spectrometer operating in EI mode at 70 eV (full scan, 50-650da). The experimental conditions were as follows: carrier gas - He; temperature program: 50 ^{o}C (isothermal for 2 min), heating rate to 175 ^{o}C at 10 ^{o}C/min, to 225 ^{o}C at 6 ^{o}C/min, and to 300 ^{o}C at 4 ^{o}C/min, final isothermal temperature: 300 ^{o}C was held for 20 min. Data were processed with the Hewlett Packard Chemstation software. All compounds were identified by their mass spectra, comparison of retention times to these of standard compounds and literature data and interpretation of MS fragmentation patterns [1-2]. Geochemical parameters were calculated using peak areas acquired with the manual integration mode.

Results

Several groups of compounds were found in the investigated extracts of the material sampled at the waste dump such as *n*-alkanes, pentacyclic triterpanes, methyl- and dimethyl-biphenyls, dibenzofurane and methyldibenzofuranes and polycyclic aromatic hydrocarbons with 2-6 rings. The aliphatic hydrocarbon group is dominated by *n*-alkanes (m/z = 71). Generally, three types of *n*-alkane distributions can be distinguished in the extracts: monomodal with long-chain *n*-alkanes (*n*-C_{23} - *n*-C_{36}) dominating, monomodal dominated by short-chain *n*-alkanes (*n*-

C_{15} - n-C_{16}) as in low-temperature coal tars and bimodal with almost the same concentrations of short (n-C_{13} - n-C_{22}) and long-chain (n-C_{23} - n-C_{36}) compounds.

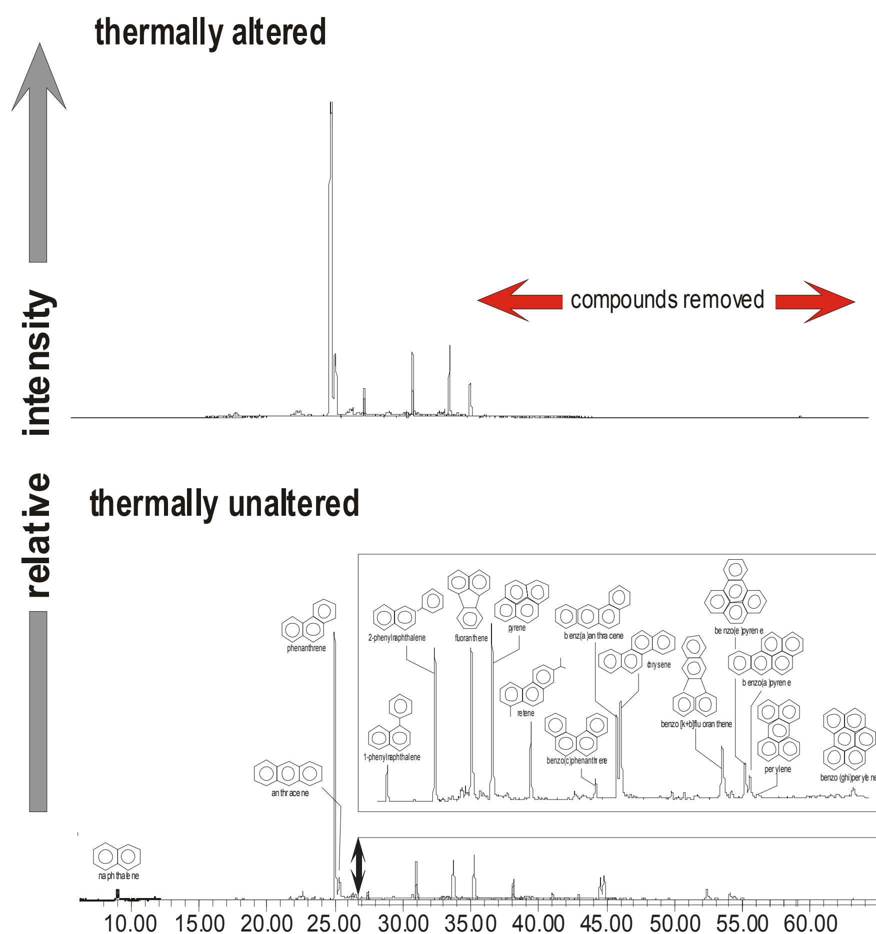

Fig. 1 Distribution of polycyclic hydrocarbons (PAHs); combined ion chromatograms m/z = 128+178+202+204+228+252+276

Pentacyclic triterpane distributions are very similar in all extracts in which these compounds occur, with the highest peak of 17α(H),21β(H)-hopane and a series of 22R and 22S diastereomers from 17α(H),21β(H)-29-homohopane (C_{31}) to 17α(H),21β(H)-29-trishomohopane (C_{33}). The uniform absence of steranes and diterpanes in all investigated extracts indicate that despite of large differences in chemical composition the most of primary

organic material of the waste showed similar geochemical properties related to their biogenic origin and depositional environment [2].

It was found that aromatic hydrocarbons distributions were influenced by waste dump fire to the high extend. Polycyclic aromatic hydrocarbons with five- and four-ring hydrocarbons do not occur in all heated samples since they show low resistance to temperature (Fig. 1). Alkylnaphthalenes and alkylphenanthrenes are also removed due to their relatively low boiling points (Fig. 2).

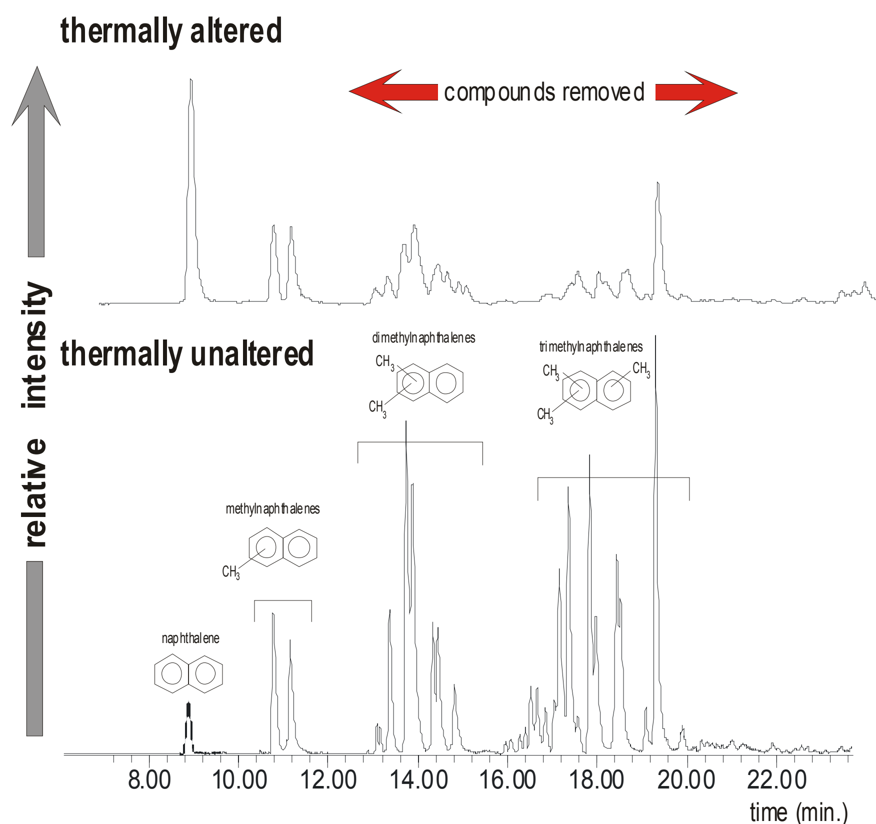

Fig. 2 Distribution of alkylnaphthalenes in organic material affected by waste dump fire; the combined ion chromatograms m/z = 128+142+156+170

To assess the extent of thermal changes in OM caused by heat several thermal maturity biomarker parameters were calculated based on aliphatic and aromatic hydrocarbons [3-4]. The best sensitivity to thermal changes and the wider range of parameter validity are shown by parameters based on aromatic hydrocarbons.

Conclusions

Chemical composition of bitumen indicates that coal waste was affected by fire in a various degree. Some samples show features relatively unaltered by temperature corresponding to beginning of catagenesis (the original coal mined in the area), in the other samples organic matter was almost completely destroyed and its maturity corresponds to the beginning of methagenesis. Less thermally stable compounds, such as lighter *n*-alkanes, cyclic isoprenoids, methyl and dimethylnaphthalenes, methyphenanthrenes and 4-5-ring polycyclic aromatic hydrocarbons were destroyed or evaporated in the case of samples which were more affected by temperature. Since thermal influence causes high variability in values of thermal maturity biomarker parameters they can be successfully applied to assess alterations in coal organic matter caused by waste dump fires.

It may be assumed that bitumen occurring in the investigated coal waste samples was formed in two different ways. It can come from organic matter present *in situ* affected by heat in various degree or be formed as pyrolysate evaporated and subsequently precipitated in colder area of the waste dump. The extracts comprise bitumen formed in one and/or both ways.

Using the boiling point values of polycyclic aromatic hydrocarbons and *n*-alkanes the temperature ranges in the sampling sites of the coal waste dump were assessed as being in the range of 280-330°C and 400-450°C.

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CHROMATOGRAPHIC CHARCTERIZATION OF ORGANIC MATTER PRESENT IN GLACIAL SEDIMENTS AND MELTWATER (SPITBERGEN)

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Introduction

Glacial erosion and sedimentation belong to one of the most important parts of rock cycle. The process rate is sufficiently high to cause considerable re-deposition of organic matter originally occurring in eroded bedrocks. In the standard method of determining suspended sediment concentration in glacial rivers it was assumed that organic matter is absent in glacial sediments. [1]. Recent work on atmospheric carbon dioxide sequestration in glacier basins states that there are no organic substances in glacial rivers of the Arctic [2]. Later findings of bacterial populations under glaciers and in the basal ice has lead to the conclusion that part of the carbon dioxide load previously sequestered as atmospheric can originate from the environment, e.g. organic matter oxidation [3]. Due to these considerations the project was undertaken to investigate a type of organic matter transported with glacial sediments and meltwater and alterations caused by transport in water. These data may also help in characterization of rocks still being covered by a glacier.

Experimental

The main set of glacial sediments and water samples was taken in July 2003, during the "Geomorphological Workshop Spitsbergen 2003", from the rivers discharging five glaciers situated along the western coast of the main island of Svalbard archipelago - Spitsbergen: Austre Bröggerbreen, Waldemarbreen, Ebbabreen and Hansbreen. Additional water and sediment samples were taken by J.Rehak from Czech Speleological Spitsbergen Expedition in September 2003 in the basin of Werenskioldbreen (Hornsund fjord region). Sediment samples from Scottbreen basin were taken by S. Bartoszewski in August 2002. Both sediment and water sampes were stored in the glass jars and transported to Poland in a cold room on the board of research vessel "Horyzont II". Extraction and determination of organic compounds was done in laboratories at the Faculty of Earth Sciences, University of Silesia.

The glacial sediment samples of about 1,5 kg were air-dried and next solvent extracted with dichloromethane in an ultrasonic bath (3 times, ultrasonification time 30 min each) to yield low-molecular organic matter. Organic matter was separated from glacial water samples (about 1,5-2,0 dm^3) by solid phase extraction (SPE) using BAKERBOND C18 speed discs. The Bakerbond Application Note EN-19 (*Extraction of PAHs from drinking water*) was applied for extraction procedures. Organic compounds were eluted from discs with several portions of dichloromethane.

Composition of extracts was investigated with gas chromatography mass spectrometry (GC-MS). An Agilent Techn. gas chromatograph was equipped with HP-17 column (60m x

0.25mm), coated by 0.25 μm stationary phase film. The gas chromatograph was coupled to a mass spectrometer operating in EI mode at 70 eV (full scan, 50 - 650 da). The experimental conditions were as follows: carrier gas - He; temperature program: 50ºC (isothermal for 2 min), heating rate to 175ºC at 10ºC/min, to 225ºC at 6ºC/min, and to 300ºC at 4ºC/min; final isothermal temperature of 300ºC was held for 20 min. Data were processed with the Hewlett Packard Chemstation software. All compounds were identified by their mass spectra, comparison of retention times to these of standard compounds and literature data and interpretation of MS fragmentation patterns [4-5].

Results

The following compound groups were identified in glacial sediment and water extracts: *n*-alkanes, *n*-alkenes, acyclic isoprenoids (mainly pristane i phytane), diterpenes, hopanes, steranes, sterols, aromatic hydrocarbons with 1-5 condensed rings substituted with aliphatic groups, methylbiphenyls, dimetylbiphenyls, phenol derivatives, oxygen and nitrogen heterocyclic compounds, and carboxylic acids.

There are five different types of *n*-alkane distributions related both to primary origin of organic matter and water washing influence: monomodal dominated by short-chain *n*-alkanes, monomodal dominated by long-chain *n*-alkanes with or without odd-over-even carbon number predominance (both show significant water washing) and two bimodal types with different contents of short- and long-chain *n*-alkanes.

Two extreme types of hopane distribution corresponding and mixtures of them in different proportions occur in the extracts (Fig. 1). One of hopane distribution types corresponds to immature (early diagenesis) (a) while the second to more mature organic matter (the beginning of catagenesis at least) (b).

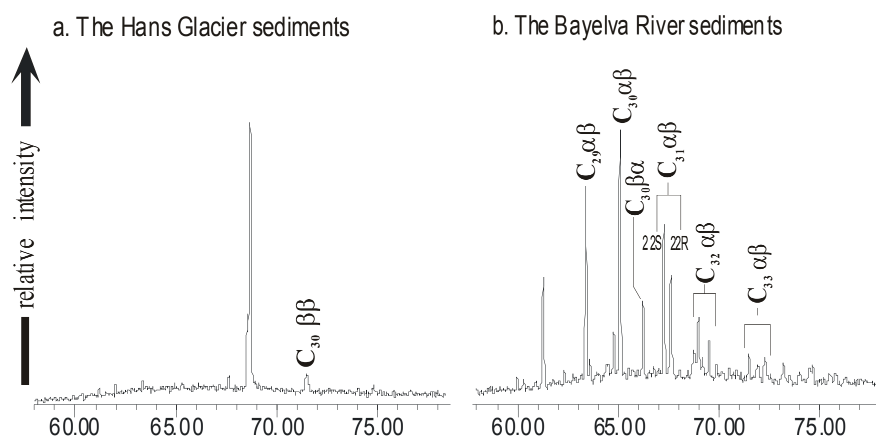

Figure 1 Two extreme types of hopane distribution

Steranes occur in meltwater samples and only two sediments from the Scott Glacier (Scottbreen). Two distributions have been found differing in ergostane (C_{28}) concentrations

(Fig. 2). Sterane distribution in all samples corresponds to advanced catagenesis. Sterols were found in several glacial sediments and their distribution rich in stigmastane (C_{29}) derivatives indicate vascular plants as their origin.

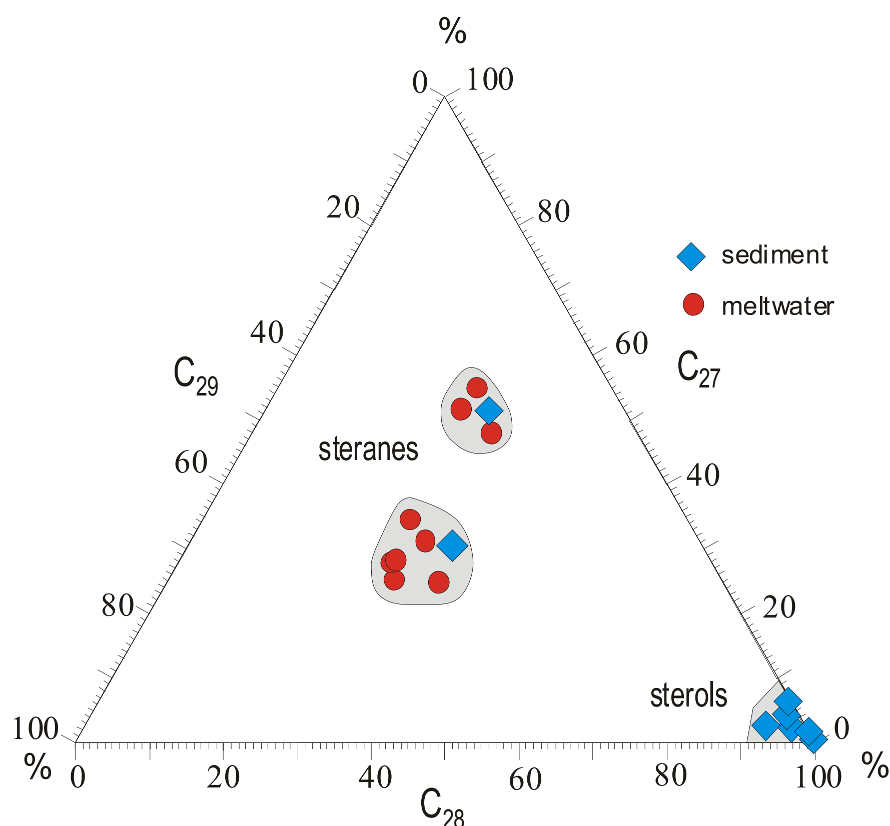

Fig. 2 Relative concentrations of steranes and sterols in meltwater and sediments

Several geochemical parameters based on biomarkers defining thermal maturity and biogenic origin/depositional environment were calculated to assess biological origin of organic matter, its stage of water washing and thermal maturity [5].

Conclusions

It was found that water washing affected only limited number of organic matter found in glacial meltwaters and concerns mainly methylnaphthalenes and lighter *n*-alkanes showing typical "cut" *n*-alkane outline. The discrepancies in values of thermal maturity indices and the presence of biochemical precursors and their geochemical products (e.g. steranes and sterols, hopanes and moretanes) testify that there are two main types of organic matter in the investigated samples, both sediments and waters:

1) organic matter of relatively low thermal maturity being at early diagenesis; it may be assumed that this organic matter comes from tundra vascular plants of recent or nearly recent age;
2) organic matter of more advanced maturity, different in sediments from different parts of the glacier, however higher than R_r = 0,50% (the beginning of catagenesis) in all samples. Its biological origin is variable, possibly algal with significant vascular land plants input [5]. Conifers did not participate in formation of the primary organic material in this case since only low concentrations of diterpenes occur.

It may be assumed that organic matter present in meltwater does not come from the same host rocks and glacial sediments due to its much higher thermal maturity and different distribution of biomarkers investigated.

Acknowledgements:

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DETERMINATION OF ACYLGLYCEROLS IN DIESEL OILS BY GAS CHROMATOGRAPHY

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Key words: FAME (*Fatty Acid Methyl Esters*), acylglycerols, diesel oil, GC

Abstract

In many EU countries and outside EU, besides the pure methyl esters B-100, also mixtures of methyl esters are added to diesel fuel. To be used as fuel methyl esters must meet certain requirements, one of which is a certain level of acylglycerols. The paper presents results of determination of acylglycerols in diesel oil dotted with FAME. The compounds were determined by gas chromatography using a high-temperature capillary column DB-5HT, made by J&W, and 1,2,3-tricaproylglycerol as internal standard.

Introduction

The problems related to the use and production of biofuels have become of increasing importance in view of the consequences of the fact that at present transport practically in 100% depends on petroleum and brings the greatest contribution to the global and local pollution of the environment. The necessity for changes has been reflected in the Directive of the EU Parliament 2003/30/EC of May 8th, 2003 in which it is recommended that EU countries should introduce the addition of biofuels in the amount of 2% till 2005, 5.75% till 2010 and 20% till 2020.

Assessment of the suitability of esters produced from vegetable oils (FAME) as fuel is based on the determination of the content of mono-, di- and triacylglycerols along with free and total glycerol. Their content is one of the most important factors determining the quality of the esters and their possible use as fuel components. The method of glycerine and acylglycerols analysis in the products of transesterification is given in the norm EN-14105 [1], unfortunately, it applies to pure FAME and can be performed prior to the mixing with diesel oil. Although experimental methods for testing the quality of the mixtures of FAME and diesel oil have been proposed [2], they do not permit determination of acylglycerols content. The method proposed in this work enables a determination of the contents of acylglycerols in diesel oil dotted with methyl esters labelled as B5, B10, B20 and B50. The determination has been made by gas chromatography with a capillary column enabling separation in high-temperatures DB-5HT made by J&W using 1,2,3-tricaproylglycerol (tricaprin) as internal standard. Prior to the analysis the components had been subjected to derivatisation by N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA).

Analytical part

The range of application

The method is to be applied for determination of mono-, di- and triacylglycerols in diesel oil admixtured with biofuel components of fatty acid methyl esters. It permits controlling the content of the biocomponent after having been mixed with diesel oil, which is the main advantage of the method over that proposed in EN-14105, allowing determination of the acylglycerols only in pure FAME.

Equipment

a) Gas chromatograph equipped with a flame ionisation detector (FID) and cold on-column injector (HP 5890 series II)
b) Capillary column with a non-polar phase of 95%-dimethyl-5%-diphenyl polysiloxane (we used a DB-5HT column made by J&W of 15m in length, internal diameter of 0.32mm, coated with a stationary film phase of 0.1μm in thickness).
c) Microliter syringes of 10 mm^3 in capacity with fused silica needle (0.18mm OD., 11.5cm length)
d) Screw-cap vials with PTFE – faced septa of 10cm^3 capacity, microliter syringes of 100 mm^3 and precision pipette of 1cm^3 capacity.

Standards

The standards, n-heptane and MSTFA used were analytical grade purchased from Supelco and Aldrich.

Chromatographic analysis

Chromatographic analysis was performed on a capillary column DB-5HT at the carrier gas (helium) pressure of 50kPa. The temperature programme of the analysis included: 50°C held for 1 min, programmed at 15°C/min up to 180°C, programmed at 7°C/min up to 230°C, programmed at 10°C/min up to 390°C, final temperature held for 5min. The injector and detector temperature was 380°C. The amount of the sample injected on the column was 2μl.

Preparation of standard solutions and analyses by interpolative internal standard method (IISM), determination of mono-, di-, triacylglycerols in diesel oil.

Calibration solutions. The following solutions of the standards in pyridine were prepared: monoolein at 5000μg/cm^3; diolein at 5000μg/cm^3; triolein at 5000μg/cm^3 and the internal standard of tricaprin at 8000μg/cm^3. At the next step 6 calibration solutions containing the above-mentioned solutions in the amounts given in table were made and placed in vials closed with PTFE membranes.

Table 1. Composition of the calibration solutions

Calibration solution	0	1	2	3	4	5
µl monoolein solution	0	20	50	100	150	200
µl diolein solution	0	10	20	40	70	100
µl triolein solution	0	10	20	40	70	100

To each calibration solution the following portions: 100 µl of the internal standard - tricaprin, 100 µl of diesel oil and 100 µl of MSTFA, were added and the contents were shaken vigorously. After 15 minutes heptane was added to a volume of $1.2 cm^3$.

Preparation of a diesel oil sample. A portion of 100 mg of diesel oil admixtured with FAME was placed in a vial closed with a PTFE membrane. Then, 100 µl of the internal standard and 100 µl of MSTFA were added and the vial was closed and shaken to mix the components. After 15 minutes 0.9 cm^3 of heptane was added.

Identification

The compounds studied were identified on the basis of the relative retention times, measured with respect to the internal standard. The times were determined on the basis of those obtained from analysis of the calibration solutions. The relative retention times are given in Table 2.

Table 2. Relative retention times

Compound	Relative retention time
Monoacylglycerols	0.71
Tricaprin	1.00
Diacylglycerols	1.18
Triacylglycerols	1.45

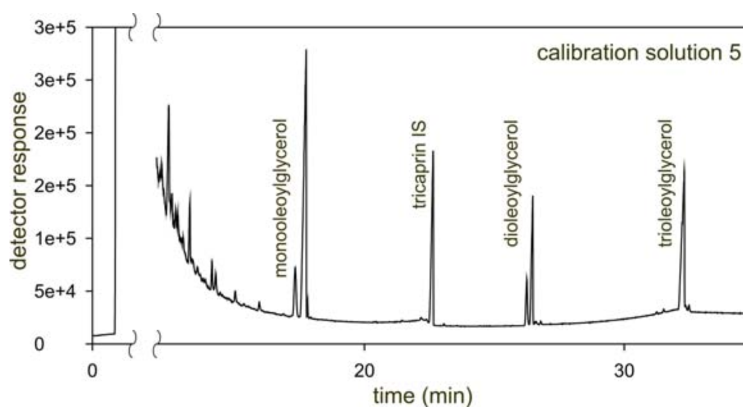

Fig 1. Chromatogram of the calibration solution

Calculation of the regression parameters of the calibration curves.

Using the calibration solutions the calibration curves were determined for mono-, di- and triacylglycerols. For the linear fragments of the curves the regression analysis was made in order to establish the parameters of the regression equation $y = ax + b$,
where: $y = M/M_{is}$; $x = A/A_{is}$;
M – the mass of acylglycerol added,
M_{is} – the mass of the internal standard,
A – the area under the peak obtained for a given acylglycerol,
A_{is} – the area under the peak obtained for the internal standard.

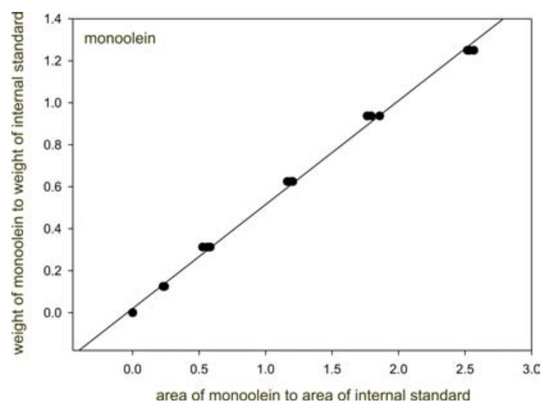

Fig. 2 The ratio of the area of the peak corresponding to monoacylglycerols to that of the peak corresponding to the internal standard versus the monoacylglycerols concentration.

The regression line parameters are given in Table 3.

Table 3. Linear regression parameters calculated on the basis of the ratio of the area under the peak corresponding to a given acylglycerol to that of the peak corresponding to the internal standard. Standard deviation is given in parentheses.

Compound	a	b	r (correlation coefficient)
Monoacylglycerols	0.49561 (0.00483)	0.01938 (0.00345)	0.9974 (0.00087)
Diacylglycrols	0.74173 (0.00866)	0.00235 (0.00221)	0.9971 (0.00225)
Triacylglycerols	0.56904 (0.00640)	0.00980 (0.00176)	0.9911 (0.00355)

Calculation of the contents of particular acylglycerols in the diesel oil admixtured with FAME

Percent contribution of mono-, di- and triacylglycerols in the diesel oil sample was found from the formula (1):

$$G_x\% = [a\,(A_x/A_{is}) + b]\,(M_{is}/m)\,100 \qquad (1)$$

where:

A_x – area under the peak assigned to particular acylglycerol (x: M – monoacylglycerols, D – diacylglycerols, T – triacylglycerols),
A_{is} – area under the peak assigned to the internal standard,
M_{is} – mass of the internal standard,
m – mass of the diesel oil sample.

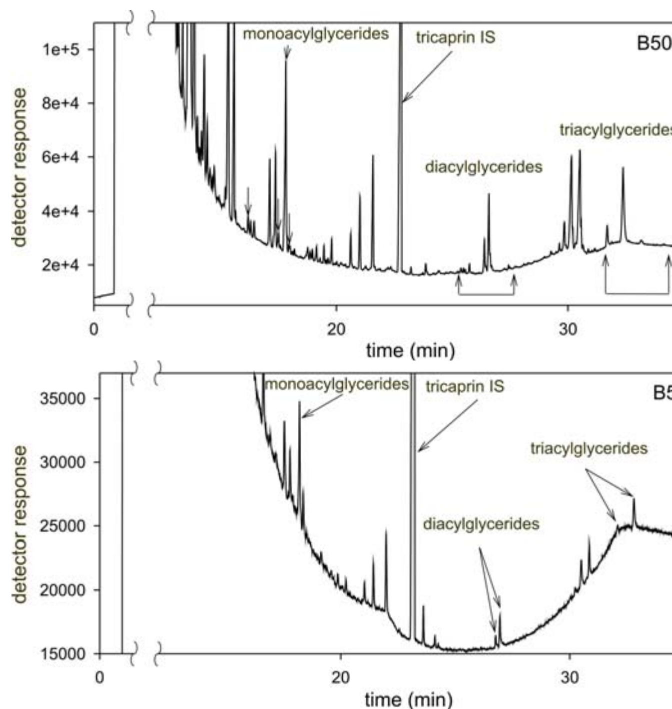

Fig 3. Chromatograms of the diesel oil analysed B-5 and B-50.

Detectability and determinability

The limits of detectability and determinability of monoacylglycerols in diesel oil were established as 0.001% and 0.003%, respectively, for diacylglycerols – as 0.0002% and 0.0007%, respectively and for triacylglycerols – as 0.0007% and 0.002%, respectively.

Repeatability

The repeatability of the method proposed was assessed on the basis of the calculated standard deviation values of content determinations of particular acylglycerols in the diesel oil containing the biocomponent. The measurements were performed for four series of six independent analyses. Measurements for each series were made on the same day. The results are given in Table 4. The repeatability estimated as a relative standard deviation varies between 0.03 and 0.09%.

Table 4. Concentrations of particular acylglycerols in a sample of diesel oil admixtured with FAME (B50) – estimation of the method repeatability.

	Series number	Acylglycerols determined					Mean	Standard deviation	RDS
Monoacyl-glycerols	1	0.1578	0.1633	0.1703	0.1759	0.1605	0.1656	0.0074	0.0448
	2	0.1598	0.1504	0.1570	0.1598	0.1624	0.1579	0.0046	0.0290
	3	0.1614	0.1511	0.1575	0.1593	0.1507	0.1560	0.0049	0.0312
Diacyl-glycerols	1	0.0918	0.0936	0.0833	0.0911	0.0866	0.0893	0.0042	0.0472
	2	0.0893	0.0878	0.0880	0.0845	0.0763	0.0852	0.0053	0.0617
	3	0.0870	0.0914	0.0825	0.0924	0.0895	0.0886	0.0040	0.0447
Triacyl-glycerols	1	0.1020	0.1032	0.0999	0.0983	0.0969	0.1001	0.0026	0.0259
	2	0.1148	0.1077	0.1022	0.0930	0.0945	0.1024	0.0091	0.0891
	3	0.1077	0.0962	0.0963	0.0968	0.1000	0.0994	0.0049	0.0491

Indirect precision

The indirect precision was estimated as the relative standard deviation calculated for all results, that is for the 3 series of 5 results. The calculations were made for the same data as those used for the assessment of the method's repeatability (Table 4).
The values obtained are presented in Table 5.

Table 5. Indirect precision calculated for particular acylglycerols in the diesel oil.

Acylglycerols	Mean	Standard deviation	RSD
Mono	0.1598	0.0069	0.0432
Di	0.0877	0.0046	0.0525
Tri	0.0969	0.0055	0.0568

As expected, the indirect precision is higher than the repeatability as the former is affected by a greater number of variables (e.g. the time of measurements).

Uncertainty

The uncertainty was calculated according to the GUM recommendations [4]. The calculations were performed by the software recommended by GUM, i.e. by GUM Workbrench provided by Metrodata GmbH [5]. The concentrations of particular acyglycerols in diesel oil were calculated by the earlier described relation eq.(1).

The calculations were performed for 6 times repeated measurements for the same B-50 oil sample.

The values of the total and extended uncertainties were estimated taking into regard the effect of the following parameters.
- Purity of acylglycerols and the internal standard,
- Concentration of the standard acylglycerols solutions and that of the internal standard,
- Volumes of the standard acylglycerol solutions and that of the internal standard,
- Peak areas assigned to the standards and the internal standard in the calculations of regression parameters,

- Mass of the diesel oil sample with biocomponents and that of the internal standard taken in determination of acylglycerols in the diesel oil,
- Peak areas assigned to the acylglycerols and the internal standard in determination of acylglycerols in diesel oil.

The values of the extended uncertainty and extension coefficient calculated by the above-mentioned software are given in Table 6.

Table 6. Values of extended uncertainty (U) and the coefficient of extension (k).

Acylglycerols	Mean	U	k
Mono	0.1570	0.014	2.3
Di	0.0893	0.011	2.3
Tri	0.1050	0.011	2.5

Accuracy

The method's accuracy was estimated on the basis of a comparison of the results obtained with the values determined by the gravimetric method in the procedure of preparation of the reference standards of the known content of methyl esters in diesel oil. Because of the lack of the certified reference material, a solution was prepared of a known content of FAME in diesel oil. The FAME used in this solution had a known content of acylglycerols. The uncertainty was also assessed for the reference standard.

The consistence of the results was verified using the following relation:

$$|x - x_{ref}| < 2\sqrt{u(x)^2 + u(x_{ref})^2} \quad (2)$$

When the relation holds, the result is assumed consistent with the reference value.

Table 7. Comparison of the results of measurements with the reference values taking into account the uncertainties.

Acylglycerols determined	Reference value, x_{ref}	Uncertainty for reference material U	$\lvert x - x_{ref} \rvert$	$2\sqrt{u(x)^2 + u(x_{ref})^2}$
Mono	0.165	0.060	0.008	0.123
Di	0.073	0.048	0.016	0.099
Tri	0.149	0.084	0.044	0.169

Conclusions

According to the norm EN-14214 [3], the admissible content of monoacylglycerols in the fuel biocomponent is 0.8%, diacylglycerols - 0.2% and triacylglycerols - 0.2%. The limits of determinability of these compounds ensured by the method proposed enable recommendation of the method for controlling the concentrations of acylglycerols in the biocomponent also in mixtures with diesel oil. The conclusion is valid for the mixtures FAME/diesel oil at the ratio of 5/95 or higher.

References

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