SESSION I

WEDNESDAY, JUNE 1st, 2016

CHAIRPERSONS: Irena Vovk

and Danilo Corradini

1.

HPTLC and HPLC analysis of phytonutrients in food samples

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Major groups of phytonutrients are polyphenols (e.g. flavonoids, phenolic acids), isothiocyanates, carotenoids, triterpenoids, phytosterols etc. This is a big group of compounds with different bioactivities, like antioxidant activity, enhancement of immune response or cell-to-cell communication, lowering blood pressure and/or cholesterol level, causing death of cancer cells, etc. Some phytonutrients are investigated because of their interaction with medicines. However, many phytonutrients are still not known to be present even in some of the most popular unprocessed food (e.g. fruits or vegetables), which we consume on a daily basis. Therefore, new analytical methods are needed to control food quality and safety and to provide more information about food composition, as well as to gain knowledge about new possible ingredients for functional food and food supplement products [1, 2]. Chromatographic techniques and especially their combined use and hyphenation to mass spectrometry (MS) and UV/vis spectrophotometry are indispensable tool in analysis and discovery of new of phytonutrients. Development of the methods for screening, qualitative and quantitative analysis of phytonutrients in different matrices is a base for the investigation of the composition of different foods, especially regarding the minor constituents. We will present the potential of the methods based on high performance thin-layer chromatography (HPTLC) and high performance liquid chromatography (HPLC) in qualitative and quantitative analysis of selected phytonutrients (e.g. flavonoids, carotenoids, triterpenoids, phytosterols) in food samples including food supplements. The examples will show targeted and non-targeted analyses using different stationary phases (silica gel, RP C18, diol, cellulose) and detection techniques (HPTLC-image analysis, HPTLC-densitometry HPTLC-MS, HPLC-UV/Vis, LC-MS, LC-MS^n).

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2.

Reversed phase HPLC of bioactive compounds in food matrices of plant origin: fundamental and practical aspects

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Reversed phase high performance liquid chromatography (RP-HPLC) is widely employed for the identification and quantification of biomolecules in edible plants and food matrices of plant origin. Most of these compounds are secondary metabolites produced within the plants besides the primary biosynthetic and metabolic routes. They hold various types of important functions in plant tissues, such as protection, attraction or signalling, and most of them, occurring as "non-nutritive" compounds in plant food, have found to play important roles in disease prevention and health-promoting effects. This communication discusses the results of our recent studies carried out to investigate a variety of factors that influence the chromatographic behavior of plant secondary metabolites, mainly phenolic compounds, with the purpose of developing novel RP-HPLC methods for the identification and quantification of bioactive compounds in plant extracts and foodstuff. We have investigated the dependence of retention behavior of a variety of biomolecules in RP-HPLC on the experimental parameters, such as flow rate, column length and internal diameter, dwell volume, temperature, isocratic and gradient elution mode, variation of the organic solvent concentration in gradient elution mode (gradient shape and duration). The influence of the considered parameters on the chromatographic behavior of the selected compounds has been evaluated in the framework of solvophobic theory [1], using a chromatographic modeling software that allows the development of RP-HPLC methods according to a Quality by Design (QbD) criteria, with the result of decreasing the number of experiments requested for method development and increasing flexibility in routine operations. Practical applications of the investigated approaches to the analysis of biomolecules in samples extracted from edible plants and processed food are then discussed.

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3.

Liquid chromatographic tandem mass spectrometric analysis of polyphenolic compounds in Italian spontaneous and cultivated berries: target and non-target approaches for their comparison and valorisation

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Berries of different *Vaccinium* species are widely considered important sources of polyphenolic compounds, especially anthocyanidin, in the human diet, thus providing interesting health-protecting attributes [1]. In fact, these compounds are well-known for their anti-inflammatory, antihypertensive, anti-microbial and anti-cancer properties [2].

Among *Vaccinium* berries, *V. myrtillus* (i.e the bilberry) is a wild species native to mountain areas of Northern and Central Europe, widely diffused also in Italian Alps and Apennines. In these zones the increasing presence of a different *Vaccinium* species, namely *V. uliginosum* subsp. *gaultherioides* (locally named "false bilberry") has been recently observed. The cultivation of *V. corymbosum* berries (i.e. the blueberry) is also widespread in the same area. Many studies focusing on the determination of selected anthocyanins and less frequently of other phenolic compounds, were carried out on bilberries from different European areas, as well as on various blueberry cultivars [3,4]. Nevertheless, no comprehensive investigation of the polyphenolic profiles of these *Vaccinium* species has been published to date, whereas for "false bilberry" the first information concerning its polyphenolic composition has been recently obtained by our team [5]. In this lecture the results of an in-depth comparison of the polyphenolic metabolomes of the three aforementioned *Vaccinium* species are presented. Data were obtained by coupling liquid chromatography with advanced quadrupole-linear trap-quadrupole, triple quadrupole and quadrupole-time of flight mass analysers, and were used in a Principal Component Analysis, achieving the clear separation of object scores in the principal component cartesian plane. Among the most interesting results, a general prevalence of anthocyanins in bilberry than in "false bilberry" and blueberry was highlighted, with the exceptions of (i) malvidin-3-glucoside and xyloside derivatives, and (ii) acylated anthocyanins, respectively.

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4.

The polyphenol-related EFSA health claim: A meaningful parameter for olive oil's health-promoting potential?

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In 2012, the European Food Safety Authority (EFSA) approved the health claim 432/2012, attesting olive oil polyphenols a protective effect on blood lipids from oxidative stress. A beneficial effect is obtained upon a daily intake of 20 g of olive oil, containing at least 5 mg of "hydroxytyrosol and its derivatives". However, considering the structural diversity and the encompassing mass range of hydroxytyrosol's derivatives (i.e. oleocanthal, oleuropein, ligstroside, etc.), EFSA's health claim is valid for the majority of extra virgin olive oils.

In order to prove the content of hydroxytyrosol and its derivatives in olive oil a methodology for extraction, hydrolysis and quantitative determination by means of LC-UV has been developed, optimized and tested in different laboratories.

Many polyphenols are esters that are easily hydrolyzed in the human's stomach and duodenum, yielding either hydroxytyrosol or tyrosol, which are resorbed *via* the gastro-intenstinal tract's (GIT) wall. Utilizing a parallel artificial membrane permeability assay (PAMPA), we estimated the pH-dependent GIT permeability of such health benefiting substances. Comparison is drawn between commercially available standard substances (*e.g.* tyrosol and hydroxytyrosol) and polyphenols extracted from olive oils hydrolyzed *in vitro* by gastric acid and digestion enzymes.

The results of these experiments should give a hint whether the health-promoting potential of an olive oil is best represented by a sum parameter or by direct quantification of the two most basic structural compounds tyrosol and hydroxytyrosol.

5.

Planar chromatography and chemometrics in determination of food authenticity

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Natural products extracts are complex mixtures that contain vast number of compounds. The chromatographic profile is usually taken into consideration for an assay of authenticity and quality of food. Fingerprint analysis of food samples could be defined as a set of characteristic chromatographic signals, which comparison leads to sample recognition. The entire chromatogram is treated as unique multivariate fingerprint, i.e., multidimensional vector, without special identification of single peaks. Recently, high-performance thin-layer chromatography (HPTLC) become a method of choice for such kind of studies due to the development of novel microstructured and nano monolithic stationary phases and powerful scanning and image capturing and processing devices and algorithms. It is often used as an alternative to high-performance liquid chromatography (HPLC).

Development of efficient and reliable fingerprint HPTLC method requires chemometric approach at several levels starting with application of experimental design and optimization techniques for the separation step, followed by data acquisition, and signal manipulation, and finally solving classification and modeling problem. Based on the similarity/dissimilarity analysis or correlation matrix, a number of unsupervised and supervised chemometric methods could be performed. Selection of particular chemometric technique depends on its features and the nature of a problem to be solved.

However, application of multivariate image analysis and chemometric tools for image processing and classification in thin-layer chromatography is still very poor, and in most instances, fingerprint analysis is conducted in subjective manner based on manually noted peak differences.

6.

Effect of Liquid-Solid Extraction techniques on the yield of secondary metabolites from plant material

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The extraction of phenolic compounds from plant materials compounds may be performed by traditional or by modern methods (such as: accelerated solvent extraction (ASE), ultrasound assisted extraction (USAE), microwave assisted solvent extraction (MASE) etc. The novel methods require shorter extraction time, use of low amount of solvents, allow for simultaneous parallel processing of several samples, and are automatic, but are more expensive.

The objective of this work was comparison of different extraction techniques developed for isolation of selected polyphenols from various plant materials such as: *Sambucus nigra* L. inflorescence, *Polygonum avicularae herb*, *Equisetum arvense* L. herb, *Tilia cordata* inflorescence etc.

The highest extraction yields of phenolic compounds from *Sambucus nigra* inflorescence were obtained by use of Soxhlet extraction. Ultrasonification, microwave-assisted solvent extraction, and accelerated solvent extraction result in similar extraction yield of phenolic acids. The highest extraction yields of the compounds from *Polygonum aviculare* herb were obtained by use of microwave-assisted extraction in a closed system. For extraction of ferulic acid from *P. aviculare* herb the highest yield was obtained by use of USAE, while highest extraction yields of rutin and isoquercetrin were obtained by use of ASE.

One can attempt to explain the different yields of the methods used for extraction of phenolic acids from the different plant materials. The differences might be caused by the distinct cellular structures of flowers (for *S. nigra*) and foliage (stem and leaves, for *P. aviculare*) and/or the different dimensions of the cells. Stems are largely composed of hard, mechanically resistant tissue, for example colenchyma, sclerenchyma, and elements of wood whereas leaves and flowers comprise mainly delicate parenchyma cells with large intercellular spaces. It is probable that destruction of the compact, hard structures of *Polygonum aviculare* stems and diffusion of solvent into this material requires more drastic extraction conditions. Phenolic compounds can, moreover, form strong bonds with lignin, a component of the cell walls of stems and leaves. Such lignin complexes are difficult to break down, and then classic extraction methods are less efficient. In such circumstances techniques which destroy the cell structure – USAE, ASE and MASE – result in higher yields of the phenolic compounds.

SESSION II

WEDNESDAY, JUNE 1st, 2016

CHAIRPERSONS: Danica Agbaba and Andjelija Malenović

7.

Investigation into the phenomena affecting the retention behavior of basic analytes in chaotropic chromatography

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Due to the complex mechanism underlying analytes' retention in ion-interaction chromatography with chaotropic additives, a lot of effort has been put into understanding of those systems from the various aspects. To that aim, many authors were studying and describing the influence of chaotropic ions' and organic modifiers' type and concentration, mobile phase ionic strength and stationary phase hydrophobicity on analytes' chromatographic behavior. Nevertheless, the effect of the mobile phase pH had been overlooked and studied only as factor affecting analyte's ionization. Consequently, we have studied the phenomena affecting the retention behavior of 34 structurally diverse basic drugs by systematic variation of aqueous phase pH value, sodium hexafluorophosphate concentration, and acetonitrile content in the mobile phase. Increasing pH from 2 to 4 led to longer retention times, even with analytes which remain completely protonated. An explanation for this phenomenon was sought by studying the adsorption behavior of acetonitrile and chaotropic additive onto stationary phase. It was shown that the pH value variation in the studied range led to larger surface excess of acetonitrile and consequentially enhanced adsorption of chaotropic agent, which significantly contribute to magnitude of surface potential and further longer retention times of oppositely charged analytes.

To study how analytes' structural properties influence their retention, quantitative structure-retention modelling was performed next. Chromatographic parameters and values of analytes' structural descriptors were correlated to retention data using a "mixed modelling" approach and support vector regression (SVM). The established model exhibited good predictive capabilities, incorporating three mobile phase and four molecular descriptors. While the ETA_EtaP_B_RC and XlogP can be considered as molecular descriptors which describe factors affecting retention in any RP-HPLC system, TDB9p and RDF45p are molecular descriptors which account for spatial arrangement of polarizable atoms and they can clearly relate to analytes' behavior on the stationary phase surface, where the electrostatic potential develops. Analysis of molecular descriptors forming the model provides further support to our earlier observations that the complementarity between the electronic structure of the analyte and the electric double layer developed on the stationary phase surface – represents a key structural determinant of retention in chaotropic chromatographic system.

Furthermore, we thought this issue should also be addressed with regard to the extended thermodynamic retention model that provides the most extensive consideration of the mechanisms underlying the separation in ion-interaction chromatography − contribution of the double layer formation and its electrostatic influence on the analytes retention, as well as the ion-pair formation between the chaotropic agent and the analyte. Therefore we have

developed comprehensive and readily applicable empirical retention model that includes the magnitude of surface potential changing with mobile phase pH, and the most important molecular descriptors affecting the interaction with electric double layer, as well as the ion-pair formation both in the stationary and mobile phase.

8.

Chemometric approaches for the analysis of genotoxic impurities in bulk drugs via LC-MS/MS

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Trace level genotoxic impurities (GTIs) in pharmaceutical products or bulk drugs require sensitive analytical methodologies for their analysis. The need to control most genotoxic impurities to the low ppm level presents significant analytical challenges. Such low levels usually require application of mass spectrometer (MS) detectors, which are characterized by their inherent sensitivity and remarkable selectivity. Therefore, chromatographic separation, unless needed, is not generally a requirement. However, two major conditions should be fulfilled when utilizing LC-MS methods: compatible mobile phases and avoidance of injecting extremely high concentrations of analytes. The latter constitutes a serious issue for the case of GTIs, since very high levels of the active pharmaceutical ingredient (API) should be utilized in order to obtain detectable levels of GTIs. Such condition renders efficient chromatographic separation between API and GTIs so that to direct API to waste and GTIs to MS detector via a switch valve. To this purpose application of experimental design combined with grid-point search optimization or experimental design - quality by design (QbD) approach may be used to provide suitable chromatographic conditions that can assure GTIs analysis along with MS protection. The aforementioned principles were applied to the detection of GTIs in the case of bulk meropenem and rabeprazole. Initial experiments with UV detectors were conducted helping reach efficient chromatographic separation between meropenem and its GTIs (S5, M9, 318-BP) and rabeprazole and its GTIs (CPAR, FBCI), respectively, utilizing compatible-to-MS LC conditions. Optimal conditions obtained via experimental design were transferred to LC-MS instrumentation for the validation of the final LC-MS/MS methods according to international guidelines.

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9.

Quantitative Structure – Retention Relationship modeling in green liquid chromatographic separation of selected drugs

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Liquid chromatography is generally considered not a green analytical technique as it requires organic toxic solvents for the separation. On the other side, this offers more possibilities for "greening". One of the strategies for greening liquid chromatography is the search for "green" components of the mobile phase. Acetonitrile and methanol are the most popular solvents employed in analytical HPLC, but they suffer from a number of drawbacks from the environmental point of view. The use of different types of cyclodextrin (CD) as mobile phase additives allowed us to increase the proportion of water in the mobile phases without loss in the resolution or efficiency of the separations, leading initially to a considerable reduction of the proportion of methanol and acetonitrile in the mobile phase.

A detailed knowledge of the stability and structure of the inclusion complexes between CDs and drug molecule is necessary to obtain a more detailed picture of its influence on the chromatographic process. Usually, the more lipophilic part of the molecule, which is the one responsible for the interaction with the stationary phases, is inside the CD cavity. Therefore, diminished retention times can be expected in the presence of CDs, even in mobile phases containing high proportions of water. The association constants determine drug-CD complex stability and along with concentration of CDs and other chromatographic parameters, influence retention behavior of the drugs.

This presentation is intended to give proposal how to build a *Quantitative Structure – Retention Relationship* **(**QSRR) models with a good predictive ability using molecular descriptors, drug-CD complex association constants and chromatographic parameters. The structural descriptors are usually derived by computational chemistry methods for the energy-optimized conformations and association constants could be derived from *Docking* studies. Only uncorrelated descriptors are selected to produce a QSRR equation. When CD-s is used as mobile phase additive, the driven force for separative displacement of analytes is the equilibrium between the stationary phase, the mobile phase and CD-s. As this is the new field of interest, some questions are to be answered in order to define the best choice of parameters to describe the drug-CD complex retention mechanisms in chromatographic system.

10.

Chromatographic assessment of pharmaceutical dissolution profiles

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The liberation process of a drug is the first step of its action after administration and it is (together with adsorption) the main factor responsible for bioavailability of the drug formulation. The liberation depends on the solubility of the active pharmaceutical substance and on the formulation itself (excipients, drug carriers, controlled release techniques etc.).

Analysis of this process requires registering concentration of liberated drug as a function of the time under specific laboratory conditions. The data obtained in this way is called a dissolution profile and the analysis is most frequently done chromatographically.

The equipment used to test dissolution is designed to simulate conditions inside the human stomach and it is currently normalized worldwide (the requirements of most pharmacopoeias are almost the same).

The apparatus can be based on basket, rotating paddle, reciprocating cylinder and flow-through cell, however paddle equipment is used most frequently. There is also a visible trend to normalize other requirements and considerations of testing procedure.

The obtained dissolution profile can be compared with a reference profile by various methods. A simple statistical analysis can be done with t-test and ANOVA test. More advanced model-independent methods are similarity coefficients and indices.

Finally, the profiles can be fitted to some model explaining the release process (zero order, first order, Hixson-Crowell, Higuchi, Baker-Londsale etc.) and the comparison can be done between the obtained parameters and their confidence intervals.

The presentation is intended to give a concise summary of current requirements and trends in dissolution profile testing for pharmacists and chromatoghraphers who are not familiar with pharmaceutical analysis.

SESSION III

THURSDAY, JUNE 2nd, 2016

CHAIRPERSONS: Piotr Suder

11.

Serum high-end biomarker analysis –
lipid and peptide detection in Multiple Sclerosis and Complex Regional Pain Syndrome

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Complex Regional Pain Syndrome (CRPS) is a severe and often disabling syndrome, which develops after trauma in ~5% of all cases; most often after distal radius fractures. CRPS is characterized by several clinical features including spontaneous pain and hyperalgesia. Increased neuropeptide release from peripheral nociceptors has been suggested as a possible pathophysiological mechanism triggering the symptoms. Resolvins, on the other hand, are thought to have a potent anti-hyperalgesic effect in inflammatory pain. These lipids are endogenous anti-inflammatory mediators generated during the resolution phase of acute inflammation. Another fatty acid, conjugated linoleic acid (CLA) and/or its metabolites, ameliorated autoimmune inflammation in several models of autoimmunity. In Multiple Sclerosis, immune alterations are believed to reflect an intrinsic immune dysbalance due to impaired immune-regulatory functions on the one hand and augmented proinflammatory effector cell responses on the other hand. We have employed liquid chromatography (LC)-tandem mass spectrometry methods using ion trap and Q-TOF technology to analyze both peptides and lipids in this context. In particular Ionkey (chip-based LC)-MS/MS on Synapt G2 Si proved to be very useful for the purpose.

12.

Designer drugs as the bane of modern times - non-targeted and targeted analysis of psychoactive substances in biological material

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Boosters are designed drugs of hallucinogenic, narcotic or psychoactive action. Although they appeared in the European market in the 90s, in Poland they are still gaining popularity. The emergence and presence on the market of a large number of new drugs means that analytical laboratories are working on the development of qualitative and quantitative methods applied for their analysis. Case studies will be described based on the analysis of biological materials (blood, urine, gastric content) taken from patients after ingestion of a substance from the group "NBOMe".

Identification of boosters in the material was done using high-resolution MS. Selection of precursor ion and fitting by the elemental composition (molecular formula) to a specific monoisotopic mass with an accuracy of 5 ppm has been done; usage of database to predict the likely structure of the compounds was performed. Due to the fact that by searching of compounds in selected databases (eg. ChemSpider, PubChem, Metlin) during assignment of possible structures fitted to the measured accurate mass many results (sometimes several thousands!) could be obtained, the fragmentation of ions in the auto MS/MS mode and targeted MS/MS mode was also performed. This approach resulted in limitation of the number of hits up to several times. Modification of the sample preparation stage, reduction of the MS signal suppression and the use of quantitative analysis based on weighted calibration curve with deuterated internal standard were finally evaluated. The resulting booster levels in serum varied at several tens ng/ml.

The following chemical and toxicological measurements for the routine analysis of biological material were done: immunochemistry, liquid-liquid extraction, gas chromatography with flame ionization detection (HS-GC-FID) and mass spectrometry (GC-EI-MS). Ethanol traces in urine and the presence of 11-nor-Δ9-tetrahydrokannabinolo-9-carboxylic acid metabolite in blood and urine samples were observed. The chromatographic analyses revealed the presence of new psychoactive substances in blood samples: 25B-NBOMe at the level of several tens ng/mL and 4-CMC of a few ng/mL. The high level of 25B-NBOMe in the stomach and the presence of metabolites of THC-COOH in the blood and urine samples confirmed poisoning by new boosters. Risk of acute poisoning 25C-NBOME is explained by the possibility of an overdose of very low active dose (on the order of several hundred micrograms).

13.

A metabolite profiling approach driven by automatic compound identification - Identification of detoxification mechanisms of plant secondary metabolites in insects

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Exploring the diversity of natural products in plants requires efficient methods to gain sufficient structural information to rapidly discriminate known compounds from novel or closely related ones. This process can be rendered extremely challenging when analyzing profiles of genetically-manipulated plants in which natural product biosynthetic pathways are manipulated. Especially on further trophic levels, like herbivores feeding on those plants, the consequences of these manipulations are difficult to grasp. Efficient workflows which combine statistical data mining and automatic compound identification routines are therefore needed. Here, we present a software solution for a quick and efficient metabolite profile screening to unravel the function of wild tobacco plants in which the expression of several glycosyltransferase genes has been manipulated. These genes are part of the biosynthetic pathway leading to defensive 17-hydroxygeranyllinallool diterpene glycosides (HGL-DTGs). Furthermore we analyze the next trophic level, by profiling the metabolites and their changes within the tobacco hornworm *Manduca sexta*. We illustrate the power of this software based workflow for the discovery of gene-mediated glycosylations in the HGL-DTG pathway in tobacco and their consequences in the herbivore *M. sexta*.

14.

Analysis of chosen bioactive secondary metabolites synthesized by cyanobacteria and lichens

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www.wbbib.uj.edu.pl/zaklad-fizjologii-i-biologii-rozwoju-roslin1

Cyanobacteria and lichens occurring worldwide are well known to produce a wide range of secondary metabolites with a high structural diversity. Cyanobacteria produce and may release into the aqueous environment compounds affecting taste and odour of water (e.g. geosmin), toxins (e.g. neurotoxic or cytotoxic alkaloids, hepatotoxic cyclic peptides, lipopolysaccharides) as well as linear, cyclic or multi-cyclic oligopeptides (e.g. microginins, cyanopeptolins). Although the main efforts are concentrated on monitoring of the toxins appearance, numerous cyanobacterial compounds exhibit a specific enzyme inhibition, antimicrobial, immunosuppressive and even antitumor activity and are considered as a promising source for pharmacology and industry. Great potential of lichens as producers of many bioactive metabolites such as UV-absorbing mycosporine-like amino acids is still poorly investigated. Despite the numerous analytical methods have been developed for characterization of cyanobacterial and lichens secondary metabolites profiles, the main problems are the complex matrices and low availability of standards. The aim of the presentation is to provide a brief overview of analytical procedures as well as a summarization of possibilities and limitations of instrumental analytical techniques typically employed for determination of these compounds. The examples will include analysis of cyanobacterial alkaloids, linear and cyclic oligopeptides as well as lichen mycosporine-like amino acids. The high biological activity of these secondary metabolites opens up new perspectives of their practical application.

15.

An untargeted data analysis of multi capillary column - ion mobility spectrometry (MCC-IMS) dataset from a breathomics study

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Ion mobility spectrometry combined with multicapillary column separation (MCC-IMS) is a well-known technology for detecting volatile organic compounds (VOCs) in gaseous samples. Recently, a new untargeted data size reduction strategy for analysis of MCC-IMS data was developed [1]. It comprises of wavelet transform, mask construction and a sparse discriminant analysis. It allows to reduce MCC-IMS data size from 500 000 to 50 variables relevant to the goal of analysis.

In this study, the untargeted data size reduction strategy is applied to a large breathomics dataset. The goal of the study is evaluate the effects of various respiratory diseases on breath metabolic profile. Breathomics dataset includes MCC-IMS spectra of 193 breath samples from 107 patients with various respiratory diseases (including asthma, lung cancer, COPD) and 86 controls, collected at four different sites e.g. hospitals.

A significant effect of respiratory diseases on breath profiles is revealed at two stages of an untargeted data analysis strategy: in masks of different sample classes and in results of sparse-PLS-DA models [2]. Nevertheless, a significant effect from sample location is also found. Finally, different chemometric approaches are considered to remove the influence of sample collection location on discrimination of controls and respiratory disease patients.

Acknowledgements: This research received funding from the Netherlands Organization for Scientific Research (NWO) in the framework of the Technology Area COAST.

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16.

Mass spectrometry imaging (MSI) combined with thin layer chromatography (TLC) as a technique for small molecules analysis

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Imaging mass spectrometry is an established and well-recognized technique for surface analysis. During this process, certain molecules located at a precise position on the surface, are desorbed. If only they are able to ionize under defined conditions, their *m/z* values might be detected by a mass spectrometer. It means, that the substances from the surface could be identified according to their molecular weight and verified by fragmentation. Thus, at the end, we may obtain the information about molecular composition of the analyzed surface.

Thin layer chromatography (TLC) allows for separation of complex mixtures of various substances. In the classic version of this technique, identification of the separated compounds is based on their retention factors. To estimate this value, spots characteristic for particular components are visualized using either UV - light or chemical reaction. The simplicity, cost-effectiveness and speed of TLC analysis, are the key reasons of its popularity. Nevertheless, visualization of the obtained chromatograms might sometimes be the drawback of this technique, especially when spots representing different components overlap.

Combination of simplicity and robustness of TLC analysis, with sensitivity and specificity of DESI-MS, introduces a new quality in this kind of research. It offers the possibility of unambiguous identification of almost every substance on the TLC plate, even if they are located in overlapping spots. Moreover, previous knowledge about separated sample is not necessary, since we are able to identify unknown substances present in separated sample.

Up to date, TLC/DESI-MS technique has been used for analysis of botanical samples, quality control of dietary supplements, separation of products of electrochemical reactions, and as a simple and rapid method for screening plant material for illicit substances. It could also be used as a promising strategy to obtain qualitative data on lipidome. Further progress in this technique certainly proves its usefulness for the analysis of complex mixtures.

SESSION IV

THURSDAY, JUNE 2nd, 2016

CHAIRPERSONS: Jerzy Silberring and Zbigniew Szewczuk

17.

Peptides labeled with cyclic quaternary ammonium salts for sensitive sequencing by electrospray tandem mass spectrometry

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The *de novo* peptide sequencing utilizing electrospray tandem mass spectrometry was successfully used in discovery of new peptide biomodulators and biomarkers. However, the insufficient ionization efficiency of some peptides and the resulting limited sensitivity is one of the main problems during analysis of trace amount of peptides by mass spectrometry. Therefore, the development of sensitive detection techniques for the efficient analysis of such samples by increasing the ionization efficiency of peptides is of utmost importance.

Recently, we developed an efficient method of synthesis of peptide conjugates containing various *N,N,N*-trialkylglycine moieties and applied as ionization enhancers for analysis of peptides at the attomole level using nano-LC-ESI-MRM technique.[1] Although the procedure was useful in combinatorial chemistry[2], its application in peptide sequencing is limited due to Hofmann elimination during fragmentation experiments (MS/MS)[3]. To overcome the possibility of this unwanted fragmentation we developed cyclic quaternary ammonium ionization tags, where all bonds susceptible to cleavage are protected in the form of 5- or 6-membered ring heterocycles (Fig. 1).

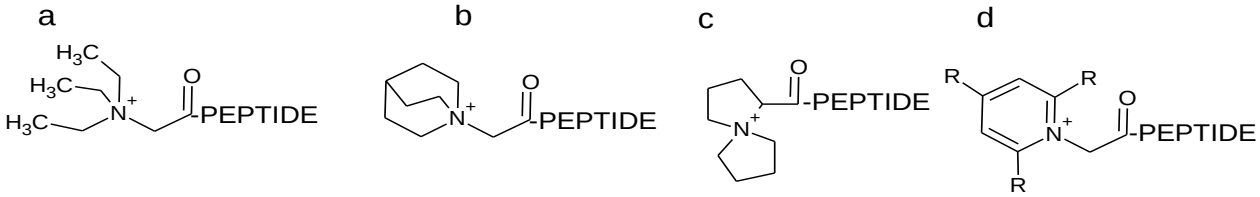

Fig. 1. Peptide conjugates containing linear (a) or cyclic ionization tags: 1-azoniabicyclic (b)[4], 5-azoniaspiro (c)[5] or 2,4,6-trisubstituted pyridinium (d) scaffolds

We also designed new derivatization reagents to introduce the ionization tags to amino or sulfhydryl groups of peptides in solution and found a method of tag conversion into their isotopologues for the quantitative research. [6] These ionization tags lower the detection limit of the analyzed peptides 10-1000 times. We believe that the application of such labeling may revolutionize comparative proteomics, leading to the development of new biomarkers based on proteins of low abundance.

Acknowledgments: This work was supported by Grant No.UMO-2013/09/B/ST4/00277 from the National Science Centre, Poland.

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18.

NGC Chromatography System – Comprehensive Solution for Protein Purification

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NGC Medium-Pressure Chromatography Systems offer a single preparative chromatography solution that scales to fit user applications and throughput requirements. The system major application is biomolecules purification.

The modular design of the NGC System provides multiple system configurations, each addressing different requirements. Each system is composed of interchangeable modules that confer different system capabilities.

Preconfigured 10 and 100 ml NGC™ Systems are available and could be easily upgradable as throughput and automation needs change — by simply add new modules.

By use the website configurator, it is possible to build own system configuration to meet specific workflow and throughput needs.

ChromLab Software is the integrated software package for the NGC™ Chromatography System. It controls all functions for laboratory-scale protein purification including instrument setup and calibration, method development, real-time monitoring and system control, chromatogram comparison, and peak analysis.

The NGC Chromatography Systems offer multiple system configurations, optional upgrades, automated multidimensional (Multi-D) chromatography workflow setup, and a common software platform for lab-scale separations. Suitable for analytical and preparative chromatography and protein purification scale-up.

19.

Fingerprints to study indole alkaloids from *psychotria nemorosa*: in extraction and fractionation optimization and in indicating interesting compounds

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Some *Psychotria* plants are used by Amazon Indians to prepare Ayahuasca, a hallucinogenic beverage. In addition, some tribes from Middle America use *Psychotria* species to treat dementia. In fact, the modulatory action of *Psychotria* alkaloid fractions and isolated compounds on enzymes related to neurodegenerative disorders was already demonstrated. One species is *Psychotria nemorosa*, which displays prominent inhibitory activity on butyrylcholinesterase (BChE) and monoamine oxidase-A (MAO-A). The extraction is a difficulty to overcome to access the plant metabolome. Several approaches have been described, mainly maximizing the number of chemical features as optimization goal. However, they may lack reliable data-handling methods. Thus, this study aims optimizing the extraction and fractionation to access the alkaloids metabolite profile of *P. nemorosa*, a source of multifunctional indole alkaloids (MIAs). Based on earlier results, ultrasound assisted extraction (UAE) was selected as extraction method. The alkaloid fraction was obtained by standardized liquid-liquid extraction (LLE), and analyzed by means of UPLC-DAD. In a first part, the extraction procedure was optimized, using a fractional factorial screening design (SD) to evaluate the influence of five factors. Two were further optimized via a central composite response surface design (RSD). Effect fingerprints were calculated and drawn for the SD. Heights of important peaks, indicated by SD, were modeled as responses. In a second part, as alternative to LLE, solid phase extraction was applied, and the fractionation was optimized in a Box-Behnken RSD, using sum of peak areas as response.

As a strategy to indicate potential MIAs, a chemometric approach was used. Forty three samples of *P. nemorosa* leaves were submitted to the optimized UAE and fractionation. The fractions were analyzed with UPLC-DAD and assayed for their BChE and MAO-A inhibition. The chromatographic fingerprints were first aligned using Correlation Optimized Warping. Principal Component Analysis was applied to explore the data structure. Linear multivariate calibration techniques, Partial Least Squares (PLS) and Orthogonal Projections to Latent Structure (O-PLS), were used to model the activities as a function of the fingerprints. The PLS regression coefficients were noisy, making the interpretation of their plot and the indication of potentially active peaks difficult. However, the O-PLS model had a lower error and an improved interpretability of the coefficients. Plotting these regression coefficients relative to the fingerprints, four peaks were indicated as multifunctional compounds, with the capacity to impair both BChE and MAO-A activities. To confirm these results, a semi-prepHPLC technique was used and a fraction containing the four peaks was purified and its activity evaluated *in vitro*. The results reinforce the prediction obtained by O-PLS modelling, confirming these four compounds as multifunctional indole alkaloids.

Acknowledgements: CNPq and FAPERGS/Brazil and FWO/Belgium for financial support.

20.

Determination of ethanol as a residual organic solvent in pharmaceutical preperation of human albumin using head space-gas chromatography-flame ionization detection (hs – gc -fid) in drug quality control laboratories

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Organic solvents play a key role in the production of pharmaceutical products during the manufacturing processes of drug substances, drug products and excipients. (synthesis of active substances ,separation, purification drying and in product formulations) . Many of them have toxic effects on humans or environmentally have hazardous properties and cannot be completely removed . Therefore, they should be avoided unless their use can be justified on the basis of risk-benefit assessmen . In order to control concentration levels of residual solvents in drug substances, products and excipients, national and international guidelines were introduced. In 1997, the International Committee for Harmonization (ICH) in their guideline Q3C [1], classified the ,commonly used organic solvents into three classes in terms of their level of hazard to humans and the environment and to regulate the concentration level of each solvent. Determination of residual organic solvents in pharmaceutical preparations has many analytical problems. The main source of problems is their high volatility and hydrophobic properties, which is directly related to the difficulty in sampling and their preparation for analysis. Moreover, the determination of polar residual solvents in pharmaceutical preparations continues to present an analytical challenge mainly because these compounds are difficult to remove from water or other polar solvents. It is a drug manufacturer and governmental quality control laboratories responsibilities to ensure that any residual solvents present in the final pharmaceutical preparation are not harmful to humans and that products do not contain levels of residual solvent higher than the recommended safety limits . Here our research is related to determination of ethanol as a residual solvent in pharmaceutical preparation of human albumin . For that purpose analytical procedure based on HS- GC – FID as analytical tool has been developed and validated . Ethanol was extracted from samples using headspace technique with extraction recoveries ranged 94 – 104 %. . Separation of ethanol was achieved on GC capillary column . For quantification analysis ,FID was used as a detector and the calibration curve was constructed and was linear with $R^2 \geq 0.9988$. The obtained validation data showed that the method appears sensitive , accurate , precise ,specific and relatively simple in both sample preparation and equipment .The procedure provides a very useful tool for routine analysis of ethanol as a residual solvent in pharmaceutical preparation of human albumin in drug quality control laboratories . In our drug quality control laboratories , human albumin samples (500 samples of pharmaceutical preparations) were tested using this method and the results showed that99.5% of the investigated samples was full fill the requirements of manufacturer and USP [2] specifications .

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Keywords : Residual Solvent in Pharmaceuticals , HS-GC- FID

21.

Enantioseparations using immobilized polysaccharide-based chiral stationary phases in supercritical fluid chromatography

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Since their introduction on the market, immobilized polysaccharide-based chiral stationary phases have been intensely investigated in high-performance liquid chromatography. These phases can be employed with a wide range of modifiers, potentially extending the application range of the polysaccharide-based stationary phases. Because an increasing number of stationary phases are being introduced in the field of chiral chromatography it is important to evaluate their enantioselectivity in different techniques.

In this study, three immobilized chiral polysaccharide-based stationary phases (Chiralpak IA, IB, and IC) were evaluated by means of supercritical fluid chromatography (SFC) with a test set of pharmaceutical racemates. First, the performance of the phases is evaluated using traditional modifiers, then with mixtures of atypical modifiers, and finally the separation results were compared with those on coated stationary phases with an equivalent chiral selector. Principal Component Analysis allowed to get a visual overview of the enantioselective patterns of the different chromatographic systems and allowed determining the (dis)similarity between individual systems. The three immobilized chiral stationary phases produced high cumulative success rates when they were screened with both traditional and atypical modifier mixtures.

22.

A novel label-free and universal detector for liquid chromatography systems using millimeter-wave technology

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Several detection techniques, such as UV-VIS spectroscopy, fluorescence spectroscopy and mass spectrometry have been employed in analytical liquid chromatography (LC) for many years. While UV and fluorescence detectors sometimes require labelling of compounds to enable their detection, MS instrumentation is universal but quite expensive. In recent years, the development of millimeter wave- and 3D-printing technology [1] has enabled the monitoring of interactions between electromagnetic waves and biological substances in micro-fluidic channels. Due to the dielectric difference between bio-molecules and mobile phases used in LC, it is possible to achieve universal detection without labelling work. In this study, a millimeter-wave sensor with operation frequency at 60 Giga-Hertz is developed and evaluated for its applicability as label-free and universal detector for a capillary liquid chromatographic system. 3D printing technology is used to fabricate the sensor structure to guarantee not only sensitivity but also to enable the microfluidic compatibility with the system as well. As a proof-of-concept demonstration, 2 UV absorbing substances (trans-stilbene oxide and praziquantel) and 1 non-UV absorbing compound (sorbitol) are injected into an open-tube setup of the LC system. The results of the millimeter wave detector are compared to that of an UV detector which is integrated in the LC instrument. The outcome shows potential of the millimeter wave sensor as an alternative label-free detector.

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SESSION V

FRIDAY, JUNE 3rd, 2016

CHAIRPERSONS: Łukasz Komsta,

Robert Skibiński and Andrzej Bąk

23.

Hyphenation of liquid chromatography and bioautography methods for analysis of antibacterial compounds in plants

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Chromatography, mostly high-performance liquid chromatography (HPLC), is a powerful tool for analyzing plant constituents. However, it does not provide direct information on their biological properties. This can be solved by thin-layer chromatography – direct bioautography (TLC-DB), that is a hyphenation of TLC with biological detection, performed directly on a developed and dried TLC plate. TLC-DB belongs to effect directed analysis (EDA) methods and may be used for searching biologically active substances even in very complex matrices. Although TLC-DB can be based on any biological effect, e.g., antioxidant, enzymatic, antiestrogenic, antibacterial, or antifungal, this term is used predominantly, when antimicrobial properties are estimated [1,2].

TLC-DB against several bacterial strains, including pathogenic and luminescent bacteria, was used as a bio-guiding method to detect substances with antibacterial activity in *Matricaria recutita* L., *Achillea millefolium* L., *Salvia officinalis* L., *Thymus vulgaris* L., *Hypericum perforatum* L. and *Chelidonium majus* L. extracts [3-5] and in their pharmaceutical preparations [6]. The targeted substances, found by TLC and TLC-DB in the analytical scale, were isolated in larger amounts using semi-preparative TLC. The isolated fractions were subjected to LC-MS/MS analysis for their structural identification.

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24.

Open screening method for analysis of biological material on the presence of medicinal drugs that have severe influence on fitness to drive

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An increasing use of drugs and mind-altering substances is a substantial challenge to toxicology laboratories, as it becomes necessary to develop targeted analytical method for the ever growing number of xenobiotics. One alternative to such methods is non-targeted screening analysis, also known as systematic toxicological analysis, which relies on identification of xenobiotics from a database of, for example, mass spectra. Its main advantage is wide applicability and relatively low analysis cost, covering mainly the purchase of equipment with an appropriately extensive database. The purchase and use of standards is not necessary, contrary to targeted analyses. The method can be easily expanded by extending the database.

Driving under the influence of drugs (DUID) is serious problem in Europe. That is why 36 institutes from 18 European countries from 2006 to 2011, participated in the Integrated Project DRUID (Driving under the Influence of Drugs, Alcohol and Medicines) to find answers to questions concerning the use of drugs or medicines that affect people's ability to drive safely. One of the aims was to gain new insights to the real degree of impairment caused by psychoactive drugs and their actual impact on road safety and develop and agree on input for the establishment of a European categorisation system for medicines and driving. In order to categorise a medicine with regard to driving, several steps are identified using pharmacodynamic and pharmacokinetic as well as pharmaco-vigilance, experimental, epidemiological and additional data (e.g. from accidentology). The DRUID expert group established and agreed that, according to its influence on the ability to drive, a medicine could, regarding to driving, be categorized as followed: category 0 (no or negligible influence on fitness to drive), category I (minor influence on fitness to drive), category II (moderate influence on fitness to drive) and category III (severe influence on fitness to drive). In our studies we focused on drugs from category III.

The goal of this project was to develop a screening method for identification drugs affecting psychomotor skills in whole blood and urine.

The screening method proposed by the equipment manufacturer was modified to include drugs influencing psycho-physical abilities, listed in the III group of substances in the DRUID project report. The supplied library was supplemented with missing mass spectra obtained from the analysis of standards. The analytical material was blood from blood donation center as with as blood and urine routinely sent to the Institute of Forensic Research for analyses. The analytes were isolated by liquid-liquid extraction with ethyl acetate (pH 11) and diethyl ether (pH 3). Samples were analysed by liquid chromatography with mass spectrometry, LC-MS/MS with linear ion trap detection (QTRAP). Analyses were carried out using a SCIEX 3200 QTRAP LC-MS/MS System. Separation was performed on a Kinetex C18 (50x3 mm, 2.6 μm particle size) column using gradient elution of water and 1% (v/v) ammonium formate in methanol and acetonitrile 1:1 mixture.

The assay was found to be selective for all tested compounds. The LC-MS/MS method has proven to be appropriate for identification of the components in whole blood and urine. It was successfully applied in day routine toxicological analysis. The procedure can be easily extended.

Keywords: systematic toxicological analysis, drug screening, LC-MS/MS

25.

The chromatographic analysis of head and neck cartilage biocompatibility

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Laryngectomy is used in the treatment of larynx cancer, serious injury or necrosis caused by radiation therapy. Laryngeal resection often results in major aspiration problems, making larynx preservation during surgical removal of tumors. To increase the comfort of patients after surgery there are attempts to reconstruct the larynx using cartilage from other parts of the body. Extra tissue fills the space where the cancerous tissue has been removed. These tissues will help fill in the spaces missing after removing the cancer. Among the sources of cartilage for reconstruction are nose (nasal septum) and external ear. In our study we present the preliminary results of the comparative analyses of the nose, ear and larynx cartilage using chromatographic methods.

To analyze nose, ear and larynx cartilage we used UHPLC system equipped with Aeris Peptide XB-C18 (150 x 2.1 mm; particle size: 1.7 μm) column. The column contained uniform porous silica layers grown around a solid, spherical silica core. The following parameters were used temperature: 30 °C sample eluent: physiologic saline concentration from: 800 ppm each component injection volume: 10 μL, mobile phase A: water B: acetonitrile; flow rate: 0.2-0.9 mL/min. Detection of the separated peaks was realized by the multiple DAD and FLD detectors enabling detection of trace-level components. Both phases were mixed with advanced micro-mixer.

The method seems to be really useful in biological (medical) material estimation in head and neck surgical reconstruction.

26.

Hybrid approach combining chemometrics and likelihood ratio for evaluation of chromatograms for forensic purposes

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The rapid development of the instrumental analytical techniques triggers the new possibilities for examination of samples of the microtrace size, which is the issue in the forensic field [1]. The advancement of the analytical techniques enables recording thousands of parameters characterising samples in a single measurement. With growing complexity of data there is an increasing need for developing the tools assisting in their interpretation, especially concerning reduction of data dimensionality. Chemometric methods, which are efficient in data compression, cannot be directly applied for interpreting the evidence data due to ignoring some aspects such as the rarity of the observed features, which is of utmost importance when interpreting the data for forensic purposes. All relevant factors are accomplished in the likelihood ratio (LR) framework, which enables reporting the evidential value of the physicochemical data in a way appreciated by the forensic community. LR ($LR=f(E|H_1)/ f(E|H_2)$) interprets the data (E) in the light of two contrasting propositions: H_1 stating that compared recovered and control materials come from the same source (e.g. suspected car), and H_2 assuming that compared recovered and control materials do not come from the same source [1]. Its only drawback lies in the fact that LR easily copes with data with the number of variables far less than the number of samples they describe and is poorly adaptable for reverse cases for highly multidimensional data delivered by advanced analytical techniques such as chromatograms [2].

The aim of the presented research was to report the evidential value of highly multidimensional data by using a hybrid models combining chemometric techniques for data compression and adopting their outcome as the input for LR models [2].

The developed models were used to solve the comparison problem of chromatograms obtained for the samples of polypropylene and analysed with pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS). Pieces of polypropylene originating from the elements of vehicles body and plastic containers are likely to be found on the scene of car accident. They were pyrolysed at a temperature 750°C for 15 s in the stream of carrier gas (helium). Obtained volatile organic compounds were transported via heated interface to GC-MS module, where they were separated and detected. The proposed LR models engaged the dissimilarity representation [3] for generating the new variables further encompassed in LR approach. Their performance was controlled by estimating the levels of false positive and false negative responses and by inspecting the empirical cross entropy curves.

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27.

New psychpactive substances (NPS) contained in "designer drugs" – chromatographic and spectroscopic analysis

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Beginning from mid-2000, on the global drug market compounds known as new psychoactive substances (NPS) or colloquially, as designer drugs started very effectively appearing. „Designer drugs" are groups of substances which are from the structure and mechanism of action point of view similar to illegal psychotropic substances or narcotics like amphetamine, phencyclidine, cannabis. Currently, among these compounds the most numerous group constitute the derivatives of cathinone, a biologically active alkaloid derived from the plant known as khat (*Catha edulis*). Lots of these substances are prohibited but some of them still not. However, possibilities of synthetic modifications of the original cathinone and their earlier obtained derivatives are enormous, so that once a given substance is put on the list of the prohibited psychotropic agents the new modified compounds almost instantaneously sprout on the market.

Due to steadily growing problem of new psychoactive compounds appearing on the market, elaboration of improved and/or novel identification and quantification methods becomes an urgent and very challenging task. Implementing an existing database with novel information on physicochemical and pharmacological properties of these compounds can facilitate rapid identification thereof by analytical chemists and toxicologists.

In this study, among others we present chromatographic and spectroscopic identification psychoactive substances contained in "designer drugs".

28.

Biopurification of air from VOC's mixture - optimization of biodegradation process

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Volatile Organic Compounds (VOCs) and odorous substances pose a significant part of hazardous pollution. The biogenic sources of VOC are related with occurrence tropical and coniferous forests, the vegetation processes and a number of physical activities such as volcanic eruptions or fires as well. Notwithstanding, the major cause of the environmental air pollution is anthropogenic activities. VOCs are commonly produced by a variety of chemical and petrochemical industries. Some VOCs may be harmful to human health in the long – term exposure and provoke serious illnesses. For example, sore throats, feelings of tiredness and dizziness, asthma, immune and reproductive system problems, mutagenic or carcinogenic effects. For an environmental point of view, VOCs lead to increased amount of ozone at troposphere thereby contribute to photochemical smog and the greenhouse effect [1-3].

There are plenty of methods for polluted air treatment and removal. The biologically based methods raise interest due to exploitation of natural ability of microorganism to degrade pollutants. They are environmentally friendly, without providing post – processes waste. The VOC's biotreatment carried out in the Compact Trickle Bed Bioreactor (CTBB) based on Know How of Ekoinwentyka has become an attractive alternative for many physical and physicochemical methods of air purification. Basically, this method has many advantages. The main pros include low pressure and low temperature of the biodegradation process, friendliness to human beings and surrounding environment, lack of secondary waste and low operating costs [4].

The aim of this work was biopurification of air stream from styrene, ethyl alcohol and dimethyl sulfide mixture using a trickle bed bioreactors operating with continuous bed fid with a mineral salt solution. The primary object of the investigation was searching for the optimal operational conditions in terms of air pollutant concentration and nutrients addition for a best purification efficiency.

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Comparison of composition of the volatile fraction in comercial samples of *Cistus incanus* L

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Herbal medicine or phytotherapy (which consists in practical use of knowledge on medicinal plants and treatments with herbal agents that can replace or support conventional treatment) is becoming very popular and increasingly common [1]. Current studies confirmed an effectiveness of herbs. Among the herbs, the *Cistus* species has gained great popularity. Preparations of this plant exhibit a wide spectrum of the activities including an antioxidant, antibacterial, antiviral and antifungal activity, gastroprotective properties, and inhibition of the prostate hypertrophy process [2].

The aim of this study is to compare the volatile fraction derived from herbal samples of one species, *Cistus incanus* L., originating from different manufacturers and geographic regions. Each plant sample was subjected to hydrodistillation according to the standard procedure [3] and essential oils obtained in that way were analyzed by means of gas chromatography coupled with the mass spectrometric detection (GC/MS). In order to compare chemical composition of the tested samples, the headspace (HS) GC with the MS detection was also performed.

The commercially available *Cistus incanus L.* samples tested in our study showed significant qualitative and quantitative differences in composition of the volatile fraction. An obvious conclusion is that composition of herbal medicines can distinctly vary both in qualitative and quantitative terms, thus affecting the health benefits thereof. Thus, it is necessary to define appropriate quality requirements and analytical methods, which would allow standardization of chemical composition of herbal food products, providing a repeatable efficacy and safety of their usage.

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