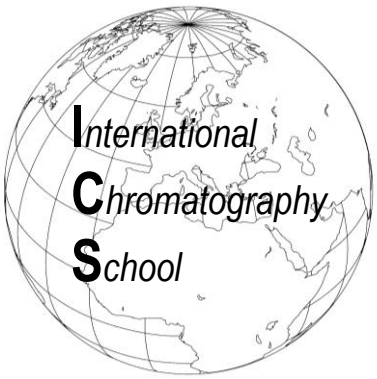
UNIVERSITY OF ZAGREB

FACULTY OF CHEMICAL ENGINEERING AND TECHNOLOGY

17th International Chromatography School



BOOK OF ABSTRACTS

6th – 7th July 2017

ZAGREB, CROATIA

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PREFACE

Welcome to 17th ICS

Thousands of scientists and engineers have worked on the development of chromatography over the last several decades. The result is one of the most versatile techniques that we have in chemical science today. The development is still going on with thousands of papers and many books being published every year. All this has been accomplished because there is an understanding of the physico-chemical principles of the chromatographic process. As an expert in chromatography each participant also needs to understand these principles and to learn how they are implemented into their daily practice.

International Chromatography School bridges the gap between the practitioner and chromatography science. It is intensive, focused on both theoretical and practical aspects of chromatography. More importantly, International Chromatography School is independent and provides an unbiased education based on scientific facts and long experience. Dissemination of knowledge plays one of the most important roles in the prosperity of particular expert, business economy of the particular company as well as for the global society. Holistically oriented policy of International Chromatography School (no registration fees) hopefully will contribute and make the difference.

President of the organization committee

Prof. Tomislav Bolanča

We would like to thank sincerely all the lecturers, sponsors and donors for their contributions at 17th ICS.

LECTURES

DETERMINATION OF HALOACETIC ACIDS IN DRINKING WATER BY ION CHROMATOGRAPHY

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Disinfection byproducts (DBPs) are a class of compounds formed through reaction of organic matter with disinfectants in the treatment process for municipal drinking water. These compounds include haloacetic acids (HAAs) that are produced during chlorination of water containing natural organic matter. Haloacetic acids are known carcinogens that have been regulated for many years. Of the nine HAAs commonly found, only the most prevalent five haloacetic acids (HAA5) are monitored. US EPA Methods 552.1, 552.2 and 552.3 used to determine HAAs require derivatization and multiple extraction steps followed by gas chromatography (GC) with electron capture detection (ECD) and mass spectrometry (MS). This presentation will describe the use of ion chromatography coupled to mass spectrometry to quantitate HAAs at low µg/L concentrations, which offers a sensitive and selective alternative that does not require sample pretreatment. Water samples are directly injected into an ion chromatograph coupled to a triple quadrupole mass spectrometer. The separation of all nine HAAs addressed in EPA methods is achieved on the IonPac AS24 anion-exchange column using a hydroxide eluent under gradient conditions. The unique selectivity of this column allows separation of these analytes from common inorganic matrix ions, so that chloride, sulfate, nitrate and bicarbonate can be diverted to waste during the analytical run and thus avoiding contamination of the ESI–MS/MS instrument. Excellent peak resolution and linearity are achieved between 0.4 µg/L and at least 20 µg/L in a high electrolyte matrix. Four isotope-labelled internal standards have been studied and the current regulatory levels (MRLs) of 1 and 2 µg/L for HAA5 are easily achieved. Similar sensitivity is observed for HAA9 targets. Recoveries of all nine HAAs are greater than 90 % in a simulated matrix.

As an alternative, the use of a heart cutting technique is described to quantitate HAAs at low µg/L concentrations. In the first channel, a standard bore column is used to separate matrix components from HAAs, which are then transferred to a concentrator column. The captured

HAAs are then separated on a capillary column with different selectivity in the second channel. The use of columns with different internal diameters yields enhanced sensitivity and the different selectivity of the columns reduces the potential for inaccurate reporting due to coelution. Both methods requires low temperature separation (15 °C), necessitating the use of an IC system capable of maintaining subambient temperature within narrow limits. With this direct injection method, recoveries for HAAs ranged from 80 – 120 % and the lowest concentration minimum reporting levels (LCMRL) were sub-µg/L, levels that are comparable to those obtained using IC coupled to tandem mass spectrometry mentioned above.

THE USE OF 2-D IC FOR THE DETERMINATION OF TRACE-LEVEL BROMATE IN DRINKING WATER WITH SUPPRESSED CONDUCTIVITY DETECTION

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Ozone is a powerful drinking water disinfectant that is effective in treating chlorine resistant organisms. For bottled water, ozonation is generally preferred over other available disinfection treatment methods because it does not leave a taste or residual disinfectant due to ozone's short lifetime. However, ozonation of drinking water containing bromide can result in the formation of the disinfection by-product bromate, a potential human carcinogen even at low μg/L concentrations.[1]

Croatia is worldwide famous for its Adriatic Sea and sea has the highest concentration of bromide (40-80 mg/L). Getting drinking water from sea water can lead to the formation of bromate if the water is disinfected by ozonization.

Determination of trace-level (low μg/L) bromate in drinking water was done on ICS-5000+ (analytical-microbore system) with two different chemistry. The first analytical column for separation and matrix elimination was Dionex IonPac AS 19 column (4×250 mm) with guard column AG 19 (4×50 mm) and the second was microbore column Dionex IonPac AS24 (2×250 mm) with guard column (2×50 mm) for 2-D determination of bromate. The sample of drinking water was concentrated on TAC-ULP1 column (5×23 mm) which was used as a loop for the second column and in this way high sensitivity of method was achieved.

Limit of quantification of method was 0.15 μg/L and recovery was at the range from 70-119 %. Large regulatory bodies (U.S. EPA and European Commission) set the maximum permissible concentration of bromate in drinking water of 10 μg/L. In Europe, therefore in Croatia, the limit is reduced to 3 μg/L for bottled natural mineral and spring water disinfected by ozonization (NN 92/2009).

References:

1. Enviromental, Determination of Bromate in Water Using Ion Chromatography, Dionex (Thermo Scientific)

HYPHENATED TECHNIQUES AND THEIR APPLICATIONS IN PLANT METABOLOMICS

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Hyphenated techniques are combination or coupling of two different analytical techniques with the help of proper interface. The term "hyphenation" was first adapted in 1980s to describe a possible combination of two or more instrumental analytical methods in a single run. Crude plant extracts represent extremely complex mixtures of numerous compounds and hyphenated techniques are widely used to solve complex analytical problems. The power of combining separation technologies with spectroscopic techniques has been demonstrated over the years for both quantitative and qualitative analysis of unknown compounds in complex plants/natural product extracts or fractions. The combined application of various hyphenated techniques even allows the discovery of new natural product, including complete and conclusive structure elucidation, and relative configurations prior to time-consuming and costly isolation and purification process. The main focus of presentation is to provide an overview of basic operational principles of various modern hyphenated techniques and to present examples of their applications in plant metabolomics.

CHROMATOGRAPHIC PURIFICATION OF MUMPS AND MEASLES VIRUS

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Mumps and measles virus (MuV and MeV, respectively) are negative strand RNA viruses and belong to the *Paramyoviridae* family. Both viruses are used for production of live attenuated vaccines used for prophylaxis of mumps and measles disease, respectively. In addition, MeV is a promising gene vector and exhibits oncolytic properties. Production of vaccines and gene therapy biologicals requires virus preparations of highest purity that enable potency and safety of the medicine. Viruses are produced using cell cultures and impurities in crude virus harvests originate either from host cells (*e.g.* host cell proteins, cellular DNA, exosomes) and cultivation medium (*e.g.* bovine serum albumin), from processing (*e.g.* extractables and leachables) or from the virus itself (*e.g.* virus aggregates, empty capsids, defective interfering particles)[1]. Purification of virus particles is still a challenging task due to the complex structure and sensitivity of the viruses[2]. Chromatography is gaining increased interest as a method of virus purification, especially monolith chromatography due to properties beneficial for viruses; mass transport based on convection, very high porosity, and high binding capacity for very large molecules. Monoliths have already been successfully used for purification and concentration of various viruses, mostly in ion-exchange chromatography.

Focus of our research is on MuV and MeV purification and to this aim we have tested immunoaffinity (IAC), ion-exchange (IEX) and hydrophobic interaction chromatography (HIC) on monolith columns. Virus purification efficiency was monitored by measuring concentration of infective particles ($CCID_{50}$ assay) and total particle concentration (Nanoparticle Tracking Analysis), host cell proteins (measured by ELISA), and genomic host cell DNA (measured by PCR). IAC was found to be very successful with recovery of infective particles around 70 %. Also, we developed a new elution system in IAC under native elution conditions enabling high recoveries of infective virus particles[3]. IAC is very specific and powerful but requires access to specific antibodies, and the use of antibodies requires more quality control which all increase costs of production. Recoveries of infective particles in IEX were quite low, only up to 20 %,

and on the other hand recoveries in HIC were around 60 %. Interestingly, total-to-infective particle ratio in the starting sample was found as an important factor in HIC recovery. Channel size of monolith columns was found important in all three investigated types of chromatography[4]. All obtained results are important for biotechnology, but also in fundamental research that requires high purity of virus particles such as study of virus proteome and lipidome.

References:

1. M. W. Wolff, U. Reichl, *Expert Rev. Vaccines* **10** (2011) 1451–1475.
2. P. Gagnon, Chromatographic purification of virus particles, u: Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation, and Cell Technology, ur. M. C. Flickinger, John Wiley & Sons Inc., 2009. pp. 1–21.
3. M. Brgles, D. Sviben, D. Forčić, B. Halassy, *J. Chromatogr. A* **1447** (2016) 107–114.
4. D. Sviben, D. Forčić, J. Ivančić-Jelečki, B. Halassy, M. Brgles, *J. Chromatogr. B* **1054** (2017) 10–19.

ANALYSIS OF SUGAR CONTENT IN SERBIAN HONEY

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Honey is the natural sweet substance produced by honey bees from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honey comb to ripen and mature. Honey is essentially a concentrated aqueous solution of different carbohydrates, including fructose, glucose, maltose, sucrose and other – oligo and – poly saccharides. The colour of honey varies from nearly colourless to dark brown. The consistency can be fluid, viscous or partly to entirely crystallised. The flavour and aroma vary, but are derived from the plant origin.[1] Serbia has a very long tradition of beekeeping. Its favourable climate, good geographical conditions and a variety of botanical species provide great potential for the development of apiculture. When placed on the market as honey or used in any product intended for human consumption, honey shall not have any other food ingredient added, including food additives, or any other additions to be made.

One of the important assessments of authenticity is sugar content, considering that natural honey primarily consists of glucose and fructose and may contain low levels of sucrose and/or maltose. Often, on the market some products could be found which are presented as honey, which consist exogenous sugars derived from cane, corn, millet or sorghum. That is one of the biggest frauds when it comes to honey.

SP Laboratorija is accredited and official laboratory for honey analysis. We can determine specific quality parameters and safety parameters. For detection of sugars: glucose, fructose, sucrose and maltose we used Ion Chromatography with Electrochemical Detector with reference silver (AgCl/Ag) electrode and working gold (Au) electrode. We used CarboPac PA10 (250×4 mm) column and a guard CarboPac PA10 (4×50 mm). Also we determined carbon and nitrogen stable isotope ratios (δ^{13}Choney, δ^{13}Cprotein, δ^{15}N) by EA-IRMS Elemental Analyzer (FlashEA 1112 HT) coupled with Isotope Ratio Mass Spectrometry (Thermo Finnigan DELTA V Advantage). This method is based on the $^{13}C/^{12}C$ carbon isotope

ratio analysis and is able to detect, with a limit of detection of 7 %, the presence of exogenous sugars derived from plants using the C4-photosynthetic cycle.

Commercial samples of different types of honey: acacia, meadow, lime, floral and forest are collected and analysed during past 5 years. In this period, we collected 24 different samples of honey, among which 6 of them were acacia type, 7 were meadow, 5 were lime, 5 were floral and 1 was forest origin.

In these samples we analysed sugar content - glucose, fructose, sucrose and the presence of C4 sugar. According to Serbian Regulation for honey, presence of sucrose cannot be above 5 g/100 g for all type of honeys, except acacia honey, where allowed content is 10 g/100 g and the sum of glucose and fructose must be above 60 g/100 g.

During these analyses, 5 samples (20.83 %) had increased sucrose content, of which 2 were meadow origin, 1 was lime, 1 was acacia and 1 was forest. According to results 4 samples (16.67 %) had lower content glucose and fructose in the sum, of which 2 were meadow, 1 was lime and 1 was acacia type. When it comes to increased content of C4 sugar, 9 samples (33.33 %) were above of 7 %, among which 3 were meadow, 3 were floral, 2 were acacia and 1 was lime origin.

According to results, we can notice that Serbian honey have appropriate quality and that generally is in accordance with Serbian and EU Regulations. In the future, monitoring for the honey should be continued with the same analysis and some new determinations (LC coupled to IRMS).

Reference:

1. CODEX Standard for honey 12-1981

NEWS IN LIQUID CHROMATOGRAPHY - SYSTEMS THAT SAVE MONEY, TIME AND EFFORT

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Method scouting systems

The optimization of analysis conditions in HPLC requires considerable time and effort. Particularly with the analysis of impurities and natural substances, methods tend to be constructed via trial and error. With conventional systems, mobile phase and column switching are performed manually by the analyst but with method scouting, multiple columns and mobile phases can be switched automatically. Method scouting analyses are performed using a variety of columns and mobile phases, to search for the optimal combination.

Method scouting system is a system with design based on the UHPLC and it provides support for efficient method development. It significantly reduces analysis preparation time and human error is reduced to the minimum thanks to automated creation of method files and batch files.

SFC/LC switching

Binary LC/SFC system can be easily configured by adding an LC pump to the SFC system. Then LC and SFC can be run on one system and this set-up saves acquisition cost as well as lab space.

The process of switching between those systems can be accomplished in about 10 min. All it takes are two blank runs using two pre-set methods which replace mobile phase and switch the method.

Method screening using both SFC and LC is the most effective to determine suitable analytical conditions quickly.

Method transfer

With method transfer systems, HPLC and UHPLC analysis can be performed with a single system, due to dual flow path for automated switching between HPLC and UHPLC.

Use of UHPLC instruments and small particles is still increasing in order to improve lab efficiency and sample throughput but lot of the analytical methods used in quality control laboratories are conventional HPLC methods. Transferring HPLC methods to UHPLC, while maintaining the integrity of the separation, requires some expertise but the use of method transfer systems allow inexperienced users to simply perform the method transfer.

SOLID-PHASE EXTRACTION – SOLUTION FOR IMPROVED BIOANALYSIS

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Modern bioanalytical laboratories are tasked with providing high-quality analytical results from complex biological samples in a high-throughput environment. The choice of sample preparation technique is important since this is the most time-consuming and labor-intensive process in bioanalysis. The importance of this step in bioanalysis steams from three major concerns – removing interferences from various biological samples matrices, concentrating the analytes of interest, and improving analytical method performance. Conventional sample preparation techniques such as dilution, filtration, liquid-liquid extraction and protein precipitation are now been consider as methods of the past.

In bioanalysis, solid phase extraction is usually chosen as one of the best options available for sample preparation as it can provide substantial benefits including low matrix factor, high sample cleanliness, and concentration of low level analytes. Although solid-phase extraction is not novel sample preparation technique, during last decade we have witnessed its rapid development in bioanalysis. Improvements in solid-phase technique have been focused on development of innovative selective sorbens such as moleculary imprinted polymers, immunosorbent, nanoparticles and multi-mode sorbents. Furthermore, formats such as dispersive solid-phase extraction, disposable pipette extraction, 96-well plates, hybrid and two-dimensional solid-phase extraction offer a variety of extraction processes and provide unique advantages of bioanalytical methods. On-line solid phase extraction utilizing column switching techniques is rapidly gaining acceptance in bioanalytical applications.

The purpose of the present work was to illustrate the importance of sample preparation exemplified by solid-phase extraction for the bioanalytical method development of drugs (immunosuppressives and chemotherapeutic agents) and vitamins.

PROTEIN AND PEPTIDE ANALYSIS BY LIQUID CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY

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One of the most popular techniques in modern protein and peptide analysis is liquid chromatography coupled to mass spectrometry (LC-MS). LC-MS based analysis has become a powerful high throughput tool for identifying and quantifying proteins and peptides in complex mixtures with high sensitivity and high resolution. LC-MS usually has large dynamic range and excellent selectivity and in the same time it is robust and easy to use. A variety of LC-MS platforms have been used for protein and peptide analysis, including coupling of off-line LC separation/spotting and matrix assisted laser desorption/ionization time-of-flight tandem mass spectrometer (MALDI TOF/TOF), and on-line LC separation and electrospray ionization (ESI) based mass spectrometer.

This lecture will cover many aspects of protein and peptide identification and targeting by LC–MS including sample preparation, methodology, instrument selection and data handling. Practical examples of proteomics experiments will be given to demonstrate discovery proteomics, proteome mapping, relative protein quantification and targeted protein quantification and identification.

REVIEW OF RESULTS OBTAINED FOR AFLATOXIN B1 IN UNPROCESSED MAIZE FROM SERBIA

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Aflatoxin B1 is mycotoxin produced by *Aspergilus flavus* and *Aspergilus parasiticus* species of mildews, which can colonize before harvest or during storage, especially after long high-humidity or drought exposure. It is the most toxic aflatoxin, in due to its very reactive epoxide metabolite which interacts with biomolecules and leads to their damage and different diseases. It is mostly found in various grains such as: maize, peanuts, cottonseed *etc.*, which are highly represented constituents in food and feed production. Also, aflatoxin B1 express its toxic effect through aflatoxin M1 – 10 times less potent metabolite – which could be found in milk, after conversion in the cow organism.

Serbia is significant producer of maize: 32nd in the World. Despite climate conditions that are suitable for agriculture, the spring and summer of 2012 in Serbia and neighboring countries were hot and very dry with prolonged drought, what was favorable for growth of *Aspergilus* species and production of Aflatoxin B1. This caused great economic losses, yet it also raised the overall awareness in terms of improving the prevention and monitoring of mycotoxins.

SP Laboratorija is official and internationally recognized laboratory, which has over 10 years' experience in mycotoxins analysis. The results obtained for aflatoxin B1 in routine samples of unprocessed maize which have been analyzed in period from 2012 to 2017, per each year separately, were evaluated in this study. We selected unprocessed maize as representative sample for the review in due to its wide and various direct and indirect usages. Analyses were carried out on high performance liquid chromatograph with fluorescent detector, according to ISO 160501.[1]

During 2012, among 461 analyzed samples of maize, 28 % of results were below the limit of quantification (LOQ), which was 0.3 ppb. The remaining 72 % of samples were contaminated, among which 12 % of analyzed samples had values above 30 ppb, which is MRL for maize according to Serbian Regulative for feed.

Total number of analyzed maize samples was double increased in 2013 (928). Among them 56 % were below LOQ while 3 % were above 30 ppb.

Total number of analyzed maize samples increased slightly during 2014 (1084) and among these samples even 73 % were below LOQ and 0.2 % were above 30 ppb.

733 samples of maize were analyzed in 2015 and 75 % of them had values below LOQ, while 1.2 % were above 30 ppb.

There were 766 total analyzed samples in 2016, among which 73 % were below LOQ, and only 1 analyzed sample was above 30 ppb (0.13 %).

Until March of 2017, 168 samples of maize were analyzed and even 82 % had values below the LOQ. The remaining 18 % were contaminated samples among which none had concentration of aflatoxin B1 above 30 ppb.

According to analyzed results, a decreasing of percentage of samples contaminated by aflatoxin B1 since 2012 can be noticed. Also, number of samples with aflatoxin B1 concentration above 30 ppb is significantly decreased. Considering this evaluation, we can conclude that level of aflatoxin B1 in Serbian maize is currently under acceptable level.

Reference:

1. ISO 16050 Foodstuffs - Determination of aflatoxin B1, and the total content of aflatoxins B1, B2, G1 and G2 in cereals, nuts and derived products - High-performance liquid chromatographic method

OPTIMIZATION OF DEVELOPER COMPOSITION FOR TLC DETERMINATION OF BINDING MEDIA ON HISTORICAL TEXTILES

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The aim of this work was to optimize a mobile phase composition for a thin layer chromatographic separation and identification of binding media on historical textile. The experiment included ultrasonic extraction of the sample, hydrolysis of referent protein materials and binding media from the textile sample, separation of amino acids in hydrolyzed sample by thin layer chromatography and densitometric determination of the components.

The optimization of the mobile phase composition was performed by means of highly sophisticated mathematical methods which are included in the State Ease Design Expert 6 and Statistica software. The goal of the optimization was to separate all present components by one dimensional thin layer chromatography. Factor of separation (RS) was chosen criteria for the optimization due to the fact that it takes into account the size of the spots as well as their position on the chromatographic plate. The result of the optimization was calculated composition of the mobile phase as: butanol : acetic acid : water = 60.14 : 18.77 : 21.09, v/v.

Parameters RF and RS calculated for the optimal composition were compared to the experimental values and they showed very good agreement. Component of binding media have been visualized by different reagents on thin layer chromatographic plates by characteristic colors. Afterwards the plates were recorded and valuated by Video densitometer. This simple and rapid chromatographic method was applied on the real sample of two hundred years old silk banner of St. Paul and St. George.

Key words: historical textile, binding media, thin layer chromatography, optimization of the mobile phase composition

HPLC-DAD-MS DETERMINATION AND CHARACTERIZATION OF CYTOCHROME P450 MEDIATED METABOLISM OF FLAVONOIDS

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Flavonoids

Flavonoids are plant products abundant in every day diet rich in vegetables and fruits. They have many salutary effects, of which their antioxidative, cardiovascular, and anticarcinogenic are the most extensively studied. This group of compounds is characterized with C_6-C_3-C_6 skeleton i.e. benzopyran ring to which phenyl is bound (Figure 1). They are divided in classes based on structure of ring C. Diversity of these compounds comes from hydroxylation and subsequent methylation and glycosylation of hydroxyl groups located on rings A and B.

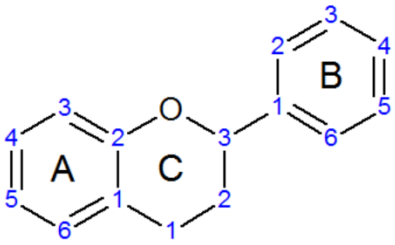

Figure 1. Flavonoid skeleton.

Cytochrome P450

Most dominant enzymes in metabolism of xenobiotics are cytochrome P450. This superfamily of enzymes catalyzes 75 % of drug metabolism. Next group of enzymes are uridinediphospho-glucuronosyltransferases. Cytochromes P450 have most significant role in phase I reactions i.e. oxidations and reductions; over 95 % of these reactions is catalyzed by P450s. These reactions are extremely diverse and include, but are not limited to, aliphatic and aromatic hydroxylations, N and S oxidations, C-N bond cleavage (demethylations, dealkylations, deaminations), C-C cleavage (aromatization), etc. Some of the most significant

cytochromes P450 that are involved in drug metabolism are CYP3A4, CYP2D6, CYP2E1, CYP2C9 and CYP2C19.

HPLC-DAD-MS

Objective of this study was to screen metabolism of 30 flavonoids using human liver microsomes and recombinant liver cytochrome P450 enzymes. For this purpose, HPLC method coupled with UV detector and MS TOF was developed. This method combined separation power of HPLC and determination of accurate mass of unknown metabolites using MS TOF. Most flavonoids could be separated based on retention time even though there are subtle differences in flavonoid structures. Structures of metabolites were elucidated using accurate mass data, retention time and were confirmed using flavonoid standards.

Results

Out of 30 flavonoids following ones have shown metabolism mediated by cytochrome P450: 3,7-dihydroxyflavone, 6-hydroxyflavone, 7-hydroxyflavone, acacetin, apigenin, flavone, galangin, kaempferol, naringenin, sakuranetin and tangeretin. Mostly hydroxylations and demethylations of flavonoid substrates were observed. The most significant enzyme involved in metabolism of analyzed flavonoids was cytochrome P450 1A2 which is in accordance with previously reported data. 7-Hydroxyflavone, acacetin and tangeretin were metabolized by cytochrome P450 2D6. Galangin and 6-hydroxyflavone were metabolized by cytochrome P450 2C19. 7-Hydroxyflavone, sakuranetin and tangeretin were metabolized by cytochrome P450 3A4.

FUNDAMENTALS AND APPLICATIONS OF SUPERCRITICAL FLUID CHROMATOGRAPHY

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Supercritical fluids are widely used as solvents for extraction in several applications because they are easy to separate from the extracted solute and leave no residue on the treated material. The advantage of using supercritical fluids in sorption processes is the opportunity to change the selectivity and capacity of the solvent simply by varying the pressure/density, which influences the solid/solute and solvent/solute interactions. Supercritical fluid chromatography (SFC) can be used as an analytical (quantitative and qualitative determination) and preparative method mostly for separation and isolation of various nutraceuticals from natural materials and purifying of pharmaceutical substances. The most common used mobile phase for SFC is carbon dioxide. The polarity of carbon dioxide at low densities is comparable to that of n-hexane and at higher densities to that of methylene chloride. SFC has several main advantages over other conventional chromatographic techniques (LC and GC). Compared with LC, SFC provides rapid separations without use of organic solvents or they are just used as additives to mobile phase. The main advantage of SFC is diffusion coefficient of solute in the SFC mobile phase, which shows to be 3 – 10 times higher than in normal liquids. In SFC, the temperature and pressure influences on mobile phase more significantly as in LC or GC. By adjusting pressure and temperature, and consequently the density and viscosity, SFC could cover wide range of applications usually obtained by LC and GC. Even through, SFC could not completely replace the other chromatographic methods due to limitation regarding to physico-chemical properties of supercritical fluids. Carbon dioxide as SCF has low critical pressure and temperature, chemical inertness and low viscosity that result in high column separation efficiencies. SFC is mainly applied within analytical or semi-preparative scales for the separations and characterization of achiral and chiral molecules, especially for bioactive compounds, like pharmaceuticals.

ACCELERATED STABILITY ASSESSMENT PROGRAM IN API DEVELOPMENT

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During the development of active pharmaceutical ingredients (API) several long-term stability studies have to be undertaken. The shelf-life, which has to be determined, is based on the time an API remains within specification limits for either potency loss or build-up of degradation products. Ensuring a stable product is of paramount importance for patient safety. Being able to find the most chemically and physically stable form during early API development, PLIVA R&D has accepted the application of an Accelerated Stability Assessment Program (ASAP). The ASAP methodology enables shelf-life prediction of APIs in two weeks using controlled miniature stability chambers and a statistical protocol. The scientific base of this methodology is a humidity-corrected Arrhenius equation employed for estimating the effects of temperature and humidity on degradation rates of APIs. This novel short-term testing method has been used on a two-component API to test the said components on mutual effects in chemical stability. The degradation products were determined by means of a RP-UHPLC with a DAD detector and a C18 UHPLC column. A chromatographic method was developed to separate all degradation products and the two APIs. Results showed an additive effect in stability of the two-component system in a two-week testing period.

HIGH-PRESSURE ION CHROMATOGRAPHY WITH THE INTEGRION ION CHROMATOGRAPHY SYSTEM

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One of the most topical subjects in conventional HPLC is the increase of sample throughput without sacrificing resolution by utilizing U-HPLC techniques. This is typically achieved by packing separator columns of shorter length and smaller internal diameter with separation materials of smaller particle sizes. However, even at optimal flow rates the resulting back pressure often exceeds the pressure tolerance of traditional HPLC hardware. Therefore, we currently witness the development of HPLC instruments with significantly improved back pressure tolerance well above 80 MPa. Although the stress on wear parts is very high at these high pressures, this development is facilitated with working materials in pumps and valves based on stainless steel.

Since ion chromatography is part of liquid chromatography, it is not surprising that a similar solution for IC is demanded as well. The fundamental difference in instrument design, however, is the fact that the fluidic pathways in ion chromatography instruments are made of metal-free components with a significantly lower pressure tolerance which excludes the use of particle sizes of around 2 µm (or smaller) typically employed in U-HPLC separations. While particle sizes of common ion-exchange materials used in analytical IC are typically around 8.5 µm, so-called fast ion-exchange columns do exist, featuring 5 µm particle sizes in smaller column formats (150 mm × 3 mm i.d.). Thus, the analysis times for anion and cation profiles could be decreased by 50 % in comparison with conventional ion exchangers. But even under these conditions, typical anion or cation profiles are characterized by a run time of around 8 min.

One possibility for further decreasing analysis times in IC is a flow rate increase beyond the van Deemter optimum, which goes along with a loss of resolution due the relatively large particle size of the ion-exchange material. Thus, this approach is only feasible for samples with a simple analyte composition and little or no matrix contamination. Doubling the linear velocity of the mobile phase through the separator column cuts the analysis time in half, while keeping

the back pressure of the separator column well below the maximum pressure tolerance of the system.

The latest development in ion chromatography hardware design is the expanded pressure tolerance of electrolytic eluent generation in capillary and analytical IC systems up to 34.5 MPa (5000 psi). This allows the use of higher linear velocities of the mobile phase in conventional ion exchangers or the use of separator columns packed with a resin of smaller particle size (4 μm). On the other hand, it also facilitates high-resolution separations of complex samples through the use of 4 μm separator columns with standard length.

The presentation will demonstrate this new trend in stationary phase design as well as some key features of the new Integrion ion chromatograph.

THC AND METABOLITES IN BIOLOGICAL SAMPLES – CHALLENGES AND REQUIREMENTS IN FORENSIC ANALYSIS LABORATORY

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Over 1000 samples of blood and urine per year are submitted to Forensic Science Center "Ivan Vučetić" for analysis. About 20 % of them are suspected to contain THC and its metabolites. Almost 70 % of THC positive samples are connected to driving impairment offenses, and the rest is left for other incidents like homicides, suicides, rapes, robberies, injuries at work etc.

Marijuana is taking big part in overall illicit drug seizures in Croatia and EU. Therefore method for analysis of biological samples containing THC and its metabolites is necessary in a forensic toxicology laboratory.

THC and its metabolites are chemically different compounds and sample preparation is a challenge. Pure THC intake is sometimes as low as 5 mg per inhalation. Blood concentration of THC is starting below 0.3 ng/mL going to 50 ng/mL, and for metabolites from 0.3 ng/mL to over 1000 ng/mL. Another limiting factor is sample amount submitted, in some cases less than 1 mL of blood/urine. THC and metabolites are thus analyzed using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) as a sensitive and selective analytical tool.

Keywords: forensic toxicology, THC and metabolites, biological samples, LC-MS/MS, extraction

BIOMIMETIC CHROMATOGRAPHIC PARAMETERS IN DRUG DISCOVERY

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The discovery and development of new drugs represents a long and an expensive process. In addition to suitable pharmacological activity and safety, a new chemical entity (NCE) has to possess a long list of adequate physicochemical and ADME (absorption, distribution, metabolism and excretion) properties to ensure its developability. In the past, ADME profiling in drug discovery was conducted in later phases of discovery, and during the 1990s this was the main reason for new potential drugs failing in clinical phases of development.[1] Therefore, it is of utmost importance to track and modify ADME properties of NCEs along with the improvement of activity towards the pharmacological target.

Chromatographic parameters are employed to investigate physicochemical properties using high-throughput and rather inexpensive methods compared to the traditional ones.[2] Lipophilicity as a key physicochemical property which influences permeability of a compound through membranes can be determined by single gradient chromatographic analysis with a previous system calibration. In a similar manner, compounds with acid-base character can be determined by three chromatographic runs at three different pH values. Further development of methodologies led to investigation of membrane interactions by employing immobilized artificial membranes (IAM-HPLC). Also, binding to plasma proteins can be studied by using human serum albumin and alpha-1-acid glycoprotein as stationary phases (HSA- and AGP-HPLC). Available methods for the measurement of biomimetic chromatographic parameters and their applications, as well as a few examples of methodology application will be presented and discussed.[3]

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INSTRUMENTS FOR POPS ANALYSIS

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Persistent organic pollutants (POPs) are organic compounds that are resistant to environmental degradation through chemical, biological, and photolytic processes. Because of their persistence, POPs bioaccumulate with potential significant impacts on human health and the environment. Humans are exposed to these chemicals in a variety of ways: mainly through the food we eat. Many products used in our daily lives may contain POPs, which have been added to improve product characteristics, such as flame retardants or surfactants. As a result, POPs can be found everywhere on our planet in measurable concentrations. The Stockholm Convention was adopted and put into practice by the United Nations Environment Programme (UNEP) on May 22, 2001. The UNEP decided that POP regulation needed to be addressed globally for the future. The purpose statement of the agreement is "to protect human health and the environment from persistent organic pollutants."

As such, POPs should be analyzed in wide variety of food, drink and environmental samples. Analysis of POPs requires instrument with high sensitivity, but also with high resolution because some of them have similar physical and chemical properties, and are hard to separate and quantify.

Good quantification needs good sample preparation. For that reason, this process should be automated. Automatization of sample preparation brings high recovery, better reproducibility, eliminates human error, and brings robustness to analysis overall. Also, one of the most important effects of bringing the instruments into sample preparation is time saving, especially if we consider that the sample preparation is usually the most time consuming step in the analytical process. In POPs analysis, automatization in the form of instruments for solid phase extraction or pressurized liquid extraction, and later on, for cleanup and concentration of extract is highly recommended.

The most commonly used technique for analysis of POPs is GC-MS. There's three instruments presented in this work. As a golden standard in a POPs and dioxins analysis, there is DFS, high resolution GC-MS. Already for decades it has been proving its proficiency in this

field of analysis and thus became the established analysis technique available nowadays in leading dioxin laboratories throughout the world. TSQ 8000 Evo, triple quadrupole system, is an extremely effective tool for routine analysis meeting all the new European Commission requirements for the confirmation of dioxins in food and feed samples. Until recently, Orbitrap technology was reserved for LC-MS, but now is also available coupled with gas chromatography. Q Exactive GC hybrid quadrupole Orbitrap mass spectrometer is an extremely effective tool for the routine screening of pesticides in food and feed samples. The Orbitrap mass spectrometer delivers excellent resolving power, mass accuracy, and sensitivity.

With all the complex challenges in the world of analysis, there is no single solution for all problems, so every alternative and different innovative approach is more than welcome.

IMPORTANCE OF CHROMATOGRAPHY IN ORGANIC SYNTHESIS

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Drug discovery and development is one of the most complex and expensive activities within the framework of the pharmaceutical industry. It incorporates a wide array of activities with a plethora of supply chain and support services. The multidisciplinary study of pharmaceutical sciences involves techniques and knowledge derived from all fields of science including biology, mathematics, physics, toxicology, and chemistry with the goal of developing, understanding and testing drugs.

The drug discovery process begins with the identification of a medical need, including a judgement on the adequacy of existing therapies (if there are any). Also, it is driven by the knowledge of chemistry of the molecules and their association with life process. The current era has witnessed an ever changing role in modern drug discovery. The chemical methods adopted for the discovery of the molecules have also undergone changes leading to the development of technologies such as combinatorial chemistry (combichem), microwave assisted organic synthesis (MAOS), flow chemistry and high-throughput (HTS) biological screening. These new technologies have enabled medicinal scientists to accelerate the discovery process.

Flow chemistry in particular has received increased attention because of its intrinsic benefits over traditional batch techniques. These include excellent heat and mass transfer for better reaction control, safe operation at high temperatures/pressures and when employing toxic or noxious reaction components, as well as automated reaction execution, purification and product isolation. Thus, increased efforts have been undertaken to incorporate flow technology into combinatorial chemistry and library synthesis.

In the context of pharmaceutical sciences, analytical chemistry is the branch of science that provides knowledge of compound separation, identification and quantification that can be useful for measuring bioavailability of drugs, purifying drugs during synthesis and identifying drug metabolic pathways. To accurately quantify drugs and metabolites in pharmacokinetic,

transport, and delivery studies, a strong understanding of analytical chemistry principles is necessary and only well-characterized analytical methods should be used to ensure the integrity of collected data. For every bench synthesis and every industrial process there are many additional steps that require characterization.

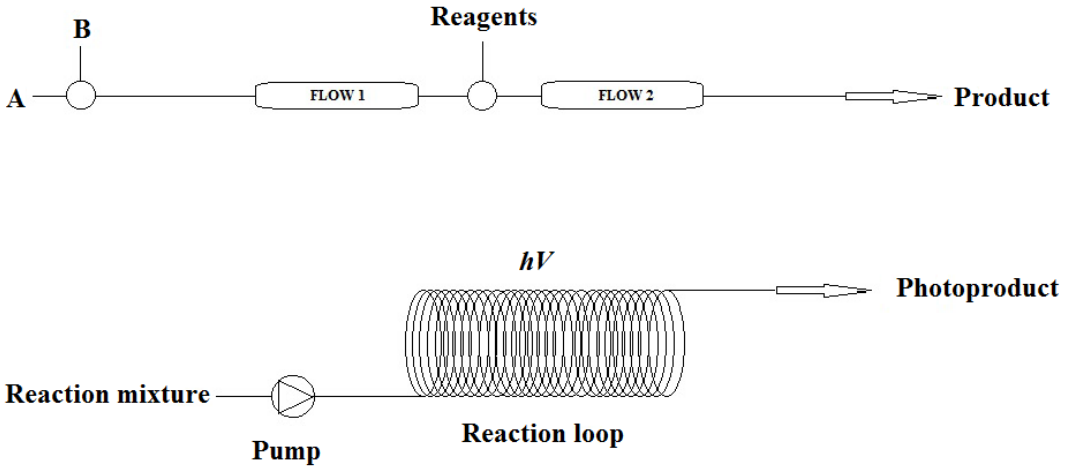

Figure 1. Flow reactor

Chromatography is a non-destructive process for resolving a multi-component mixture of traces, minor or major constituents into its individual fractions. Chromatography may be applied to both quantitative and qualitative. It is primarily a separation tool. The need for chromatographic techniques that provide increased resolution, molecular structure detection and confirmation, sensitivity and separation power, along with decreased analysis times and detection limits, has become critical for applications ranging from environmental analyses, chemical syntheses, polymer characterizations to toxicological investigations in pharmaceutical industry. To meet this need, techniques that combine various chromatographic methods, as well as ones that incorporate analytical techniques into traditional chromatography systems, have evolved.

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IS THERE A CONNECTION BETWEEN IC AND SKIING?

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Skiing industry have significant influence on the environment of ski resorts, especially on soil condition and species richness. Plants and soil on ski slopes are directly affected by slopes, changes in microclimate and vegetation, intake of water, ions and snow additives. Snow additives include chemicals such as: nitrogen-based fertilizers (urea, ammonium nitrate and ammonium sulphate, ammonium chloride), potassium chloride, sodium chloride and phosphates. The purposes of additives are to convert a soft snow to hard snow or to harden the surface of ski slope. During the last 20 years production of artificial snow increased considerably. Snowmaking begins with a water supply such as a river or reservoir and includes addition of listed chemicals. The aim of this research was to determine ionic composition of soil by means of ion chromatography method and to investigate influence of snow additives on chemical changes in soil.

The research was conducted on the Sljeme ski slope (Red Fall), which is the part of the Medvednica Nature Park. Soil sampling was conducted in April 2015 after the snow was malted on three locations on the ski slope (start line, middle of slope and finish line). The control location was located in the forest area near the ski slope. In total, 12 soil samples were taken.

Ion content in wet soil samples was extracted in ultra-pure water in 1:10 (w/v) ratio according to ÖNORM L 1092 norm. After the extraction, samples were centrifuged and filtrated in two steps: firstly through filter paper and then through cartridge (OnGuard IIP, 1cc, Dionex, Sep-Pak Vac and OASIS HLM, Waters) to prevent contamination of the separation column with organic molecules. Anions separation and detection in extract was performed on Dionex ICS-1000 system: separator column [Ion Pac AS 17 (4×250 mm) Dionex]; KOH eluent with gradient separation: (A)10 mmol L^{-1} KOH, (B) 10 mmol L^{-1} KOH – 35 mmol L^{-1} KOH – 10 mmol L^{-1} KOH in 12.5 min (C) 10 mmol L^{-1} KOH (total separation time 30 min); flow rate: 1.0 mL min^{-1}; detection: suppressed conductivity. Cation separation and detection in extract

was performed on Doinex ICS-1000 system: separator column [Ion Pac CS 16 (5×250 mm) Dionex]; MSA eluent (30 mM) constant during analysis (total separation time 30 min); flow rate: 1.5 mL min^{-1}; detection: suppressed conductivity.

Statistical analyses of differences in ion content in soil according to different locations were computed by analysis of variance (ANOVA) using SAS Institute 9.1.3 and mean values were separated by Fisher's LSD test at $P \leq 0.05$.

Results revile that artificial snow-production and chemical maintenance of snow significantly increased chloride, sodium and calcium content in the ski-slope soil compared to the ion content on the control location. The contents of fluoride, sulphate, potassium, nitrate, and magnesium on monitored locations did not differ statistically from the control location. Average values, for all four investigated locations were: 2.84 mg F^-/kg; 158.2 mg SO_4^{2-}/kg; 32.0 mg K^+/kg; 2.20 mg N-NO_3/kg and 19.8 mg Mg^{2+}/kg.

Keywords: ion chromatography, artificial snow, nitrogen-based fertilizers, soil

DETERMINATION OF CARBOHYDRATES AND AMINO ACIDS IN COMPLEX MATRICES

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Ion chromatography is one of the most intensively used methods for analysis of biomolecules such as amino acids and carbohydrates. Generally, this includes gradient separation on anion exchange resin followed by pulse amperometric detection. Gradient and pulse profile has to be optimized in order to obtain good quality results. Presence of the natural matrix makes method development even more complex process.

This lecture review and summarizes current methods for the analysis of amino acids and carbohydrates. Several examples will be presented including fermentation broth, cell cultures and tea revealing key factors within method development process.

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