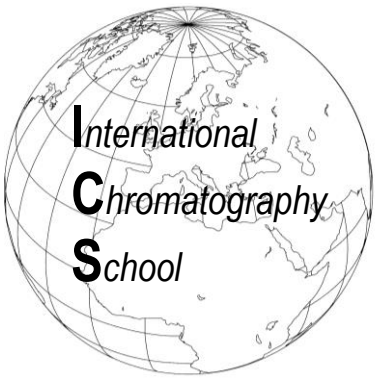
UNIVERSITY OF ZAGREB

FACULTY OF CHEMICAL ENGINEERING AND TECHNOLOGY

18th International Chromatography School



BOOK OF ABSTRACTS

14th – 15th June 2018

ZAGREB, CROATIA

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Editors:

D. Ašperger, Š. Ukić

Published by:
University of Zagreb, Faculty of Chemical Engineering and Technology

For publisher:
T. Bolanča

ISBN 978-953-6470-85-3

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PREFACE

Welcome to 18th ICS

Chromatography is one of the most versatile analytical techniques nowadays, most probably due to the fact that it simultaneously enables separation, identification, and quantification of diverse analytes, commonly in very complex matrices. Even more, numerous new inventions and improvements in a field of chromatography, followed by related applications, support significantly the continuous growth of its popularity. All this progress has been accomplished because there was an understanding of physico-chemical principles of chromatographic process. Therefore, 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 started and is still guided by idea to be a bridge that connects chromatographic theory and every-day practice; it provides an unbiased education based on scientific facts and long experience. The Organizers hope that holistically oriented policy of International Chromatography School (no registration fees) will contribute in higher accessibility of chromatographic information and thus make the difference. The School is an excellent event for those who follow the global mainstream of lifetime learning as well, providing an insight to numerous new technical solutions and trends in a field of chromatography. Last, but not least, it is very likely that solutions for many of your actual chromatographic problems can be found in some of presented lectures or that you can reach them through discussion with other school participants.

Therefore, welcome to the 18th International Chromatography School, collect the provided knowledge, meet new colleagues and make new friends!

Assoc. Prof. Šime Ukić
President of the Organizing Committee

We would like to thank sincerely all the lecturers and sponsors for their contributions at 18th ICS.

LECTURES

PRODUCTIVITY INCREASE IN PHARMA/BIOPHARMA WITH THE THERMO SCIENTIFIC™ VANQUISH™ DUO – SEE PRODUCTIVITY IN A NEW LIGHT

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As typical for many businesses, laboratories are assessed on their efficiency in terms of operations excellence, cost, quality, and response time by measuring key performance indicators (KPI). KPIs are based on the output at a certain quality per unit time. This output is balanced against related cost. Analytical laboratories must meet these demands for enhanced productivity as well as demands for use of multiple and diverse chromatography techniques, by analyzing more samples in the same time with the same amount of personnel, equipment and space, while maintaining rigorous quality standards. Highly automated laboratory equipment contributes the most to increased productivity and allows laboratories to deliver improvements in KPIs.

The Thermo Scientific™ Vanquish™ UHPLC productivity workflows address the productivity demand with a smart combination of unique dual technologies by building two flow paths into a conventional UHPLC housing. Each flow path consists of a pump, an injection unit, a column, and a detector. This Dual LC technology doubles the number of assays in the same time. Similarly, a second pump in a system can also reduce the overall analysis time by up to 60 % when used for overlapped offline column wash and re-equilibration in a Tandem LC setup. Another use is the example of a charged aerosol detector (CAD) combined with a dual gradient pump in an inverse gradient setup to quantify unknown compounds without the need for expensive standards. These three examples highlight the benefits of an UHPLC system and show how it maximizes efficiency.

RETENTION MODELING – A SHORTCUT IN DEVELOPMENT OF CHROMATOGRAPHIC METHODS

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Nowadays global awareness about human's influence on the environment is followed by various restrictive measures and more demanding legislatives in majority of developed countries, while the rest of the world is trying to follow this trend as well. Consequently, a list of priority pollutants (and their degradation products) is expending continuously, generating numerous new tasks for analytical-method developers. Also, real chromatographic samples contain matrices of diverse complexities what makes each sample specific and requires frequent modification of applied analytical methods. Application of mathematical modeling can reduce costs and experimental effort for the method development.

In this presentation, the focus will be on development of chromatographic methods, based on modeling of analytes' retention time.

Most of retention models are focused on isocratic elution, while only a few ones are considering gradient elution as well. Although, indeed, the best peak-resolution can be obtained by using isocratic elution mode, the gradient mode has some significant benefits: first of all reduction of the analysis time. Therefore gradient elution has become inevitable chromatographic approach. So-called "iso-to-grad" model is able to equally predict retention time for isocratic or gradient elution mode and, moreover, it can include matrix effect as well. Adding an appropriate peak-shape function into the model or, in addition, combining it with *quantitative structure-activity relationship* methodology, an extremely beneficial tool for optimization of chromatographic separation can be obtained.

Funding

The authors gratefully acknowledge financial support from Croatian Science Foundation through project entitled Modeling of Environmental Aspects of Advanced Water Treatment for Degradation of Priority Pollutants (MEAoWT) (IP-09-2014-7992).

ANALYSIS OF HEXAVALENT CHROMIUM AND CHROMIUM SPECIATION BY IC AND IC-ICP/MS

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Two ionic forms of chromium – trivalent chromium, Cr(III), and hexavalent chromium, Cr(VI) – are present in soil, water, and the biota. Cr(III) and Cr(VI) occur naturally in the environment, being found in water from the erosion of chromium deposits in rocks and soil. Cr(VI) is also produced by industrial processes and manufacturing activities, including discharges from steel and pulp mills. Cr(III) is a nutritionally essential element in humans and is often added to vitamins as a dietary supplement. Cr(III) has relatively low toxicity and is a concern in drinking water only at very high levels of contamination, whereas Cr(VI) is more toxic and poses potential health risks.

Chromates are oxyanions (*e.g.*, CrO_4^{2-}, $Cr_2O_7^{2-}$) of chromium in the oxidation state of +6. All Cr(VI) compounds are strong oxidizing agents that are considered to be toxic and potentially carcinogenic. Hence, chromates are regulated in the environment and are a primary drinking water contaminant in the US and in the European Union. For example, in 1999, the state of California established a public health goal (PHG) of 0.2 µg/L for Cr(VI) and 2.5 µg/L for total chromium. In July 2011, the Office of Environmental Health Hazard Assessment (OEHHA) finalized the PHG for Cr(VI) at 0.020 µg/L.

Drinking water standards are regularly re-evaluated by the US Environmental Protection Agency (EPA). Currently, dissolved Cr(VI) is measured as chromate according to US EPA Method 218.7. This method is a modified version of US EPA Method 218.6 and is based on anion-exchange chromatography. The approach uses a Thermo Scientific™ Dionex™ IonPac™ AS7 column (250 mm × 4 mm i.d.) and detection after post-column reaction with diphenylcarbazide (DPC), which yields a compound with visible absorbance at 530 nm. Using EPA Method 218.7, detection limits for Cr(VI) fortified into reagent water ranged from 0.0044 to 0.015 µg/L.

Although the PHG for Cr(VI) in drinking water in California is 0.020 μg/L, the current regulatory maximum contaminant level (MCL) is 10 μg/L. Application Update (AU) 179 describes a method that uses post-column reaction with DPC and has a method detection limit

(MDL) of 0.001 μg/L. The AU 179 method allows a minimum quantitation limit of 0.003 μg/L, which is more than sufficient for the proposed California PHG of 0.02 μg/L. Determination of Cr(VI) at the California PHG cannot be accomplished with a direct injection of drinking water, separation on a single anion-exchange column, and detection by suppressed conductivity. However, Aggrawal and Rohrer have recently shown that it is possible to design such a method to determine Cr(VI) at the 10 μg/L MCL regulatory limit.

The speciation analysis of Cr is a challenging task, because the stability of different Cr species is easily affected by conditions during sample collection and treatment. For example, low pH values may lead to the degradation of Cr (VI) to Cr (III) due to the increased redox potential, while high pH values may lead to the precipitation of Cr (III) as $Cr(OH)_3$. An additional difficulty in the accurate speciation analysis of Cr by ICP–MS are the numerous spectral interferences (*e.g.*, $^{35}Cl^{16}O^{1}H^{+}$ or $^{40}Ar^{12}C^{+}$) on the most abundant chromium isotope, ^{52}Cr.

A Thermo Scientific iCAP Qc ICP–MS was used as an element-specific detector of the Cr species eluted from the separator column. Due to the use of flatapole technology in the Thermo Scientific QCell collision cell, the iCAP Q series of ICP–MS instruments offer the selectivity to suppress spectral interferences while maintaining the high sensitivity for trace metal detection in coupled applications such as IC–ICP/MS.

NGC™ CHROMATOGRAPHY SYSTEM - DESIGNED BY YOU, BUILT BY BIO-RAD; PROTEIN PURIFICATION WORKFLOW

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The NGC family of medium pressure (MPLC) preparative chromatography systems offers a single customizable and modular laboratory chromatography solution that aligns and scales to fit the purification, automation and throughput requirements of customers. The NGC systems suit a variety of protein purification applications ranging from preparative to analytical scale, for multiple types of molecules including recombinant proteins, monoclonal antibodies, virus removals, plasma proteins for disease diagnosis and nucleic acids. Typical downstream applications for NGC customers could include a wide variety of in vitro assays, protein crystallization studies, protein-protein interaction studies and clinical studies of biotherapeutics.

The NGC platform provides a single solution that can scale and expand to customer applications and throughput requirements over time. Hardware flexibility combined with one encompassing software package provides design advantages that simplify the complexity of chromatography. From intuitive, guided plumbing and instrument set-up, experimental set-up and operation to fully integrated data analysis, the NGC systems are the next generation in protein purification.

High purity protein is a common requirement for biochemical and structural studies. A common approach is to recombinantly express an affinity-tagged version of the protein of interest. This is, however, not always a viable option. Here we discuss protein purification workflow development for untagged proteins using Bio- Rad's NGC Chromatography System and Stain-Free gel technology and introduce a new indicator of method performance, the purity quotient difference (PQD).

PRINCIPLES OF CHROMATOGRAPHY; TIPS AND TRICKS IN LC ANALYSIS

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High-performance liquid chromatography (HPLC) emerged as a powerful, versatile and sensitive technique utilized for conducting separation, identification, and quantification. According to that, the focus of the presentation will be on principles of chromatography as one of the leader techniques in modern analytical chemistry. Particular attention will be on hints and tips with troubleshooting problems related with mobile phases, columns, detectors, etc. Therefore, mainly asked questions related with troubleshooting will be discussed. For instance: how to choose the appropriate column and mobile phase, what are the usual mobile phase problems and how to prevent it, how long to equilibrate the column, what are the benefits of normal phase chromatography and when to apply it, troubleshooting with chromatographic baseline, pressure and peak retention times, how to avoid peak fronting and tailing or the split peaks, why is capacity factor one of the most essential parameters, what to do when there is a problem with analysis of polar compounds on C18 column, what is hydrophobic collapse, etc.?

DEVELOPMENT OF LC-MS/MS METHOD FOR THE ANALYSIS OF ILLICIT DRUGS AND ITS APPLICATION IN WASTEWATER-BASED EPIDEMIOLOGY

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In recent years, a new approach for estimation of drug abuse, known as wastewater-based epidemiology (WBE), has been developed. This approach is based on the analysis of the specific human urinary biomarkers in wastewater as indicators of illicit drug consumption and is increasingly used as a complementary tool to conventional methods, which are based on information about seizures, surveys on illicit drugs and number of treated drug addicts. For that purpose, an analytical method based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the analysis of 13 urinary biomarkers of illegal drugs (heroin, cocaine, cannabis, amphetamine, methamphetamine, 3.4-methylenedioxyamphetamine (MDMA)) and therapeutic opioids (methadone, codeine) in municipal wastewater was developed and validated. Different columns (Synergy Polar, Kinetex C18 and Kinetex PFP), eluents and gradient programs were evaluated to optimize chromatographic separation. At the final conditions, a complete chromatographic separation of all analytes was achieved by using Synergy Polar column with methanol and water, both containing 0.1 % acetic acid, as eluents. The selectivity of the method was assured by highly specific MRM (multiple reaction monitoring) detection, using the two most abundant precursor/product ion transitions for each analyte. Method was developed and optimized for both dissolved and particulate fraction. The enrichment of illicit drugs from the suspended solids and aqueous samples was performed employing pressurized liquid extraction (PLE) and solid-phase extraction (SPE), respectively. The performance of different SPE cartridges was investigated in order to optimize the overall recovery and to reduce the matrix effects and the optimal results were obtained by using mixed-mode cation-exchange (Oasis MCX) cartridges. The recoveries were in the range from 60 % to 94 % for the dissolved fraction, while recoveries for the particulate phase were a bit lower (21–92 %). The developed method was applied to study spatial and temporal consumption patterns of illicit drugs in Croatia. Results showed that cannabis, cocaine and

heroin are the most widely abused drugs, while recently a significant increase in consumption of amphetamine-type drugs was also observed.

Keywords: illicit drugs, liquid chromatography-tandem mass spectrometry, wastewater-based epidemiology, wastewater

ANALYSIS OF POLAR PESTICIDES IN WATER AT LOW NG/L LEVELS BY ION CHROMATOGRAPHY COUPLED TO TRIPLE QUADRUPOLE MASS SPECTROMETER

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Polar pesticides have become, in recent years, more interesting components for analysis. Increased prevention with long term effects for weeds growing along roads and development of genetically modified crops resistant to glyphosate and glufinosate helped develop a broad spectrum of herbicides and increased their use. New herbicides could possibly have potential for carcinogenic activity, although the latest research speaks against the above. The analysis of glyphosate and other polar pesticides is a new challenge. Their polarity does not permit direct analysis by HPLC without previous derivatization or use of specific columns. The above methods have shown a lack of robustness and provide questionable results. The development of ion chromatography (IC) and mass spectrometry (MS), namely triple quadrupole MS/MS, provides many advantages for the analysis of highly polar compounds. Ion chromatography is a preferred way of separating polar ionic analytes such as anions, cations or small polar molecules (metabolites) and sugars. MS/MS analysis offers low detection limits and high selectivity detection in SRM mode. IC-MS/MS provides quantitative analysis of polar pesticides including carefully monitored glyphosate and its metabolite AMPA in water matrices. Thanks to direct injection without the long and hard preparation of the samples, it enables higher speed, reliability and reduced errors when manipulating the samples.

AGRICULTURE IMPACT ON GROUNDWATER QUALITY

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Intensive agriculture is putting great pressure on the environment and therefore it is vital to rationalize its inputs, and to control and monitor the impact agricultural activities have on soil, water, and air, in order to achieve sustainability. For better understanding of the impact that conventional agricultural practices have not only on soil, but on water resources as well, two main objectives were established. The first one is to measure concentrations of nitrate (NO_3^-) and sulfate (SO_4^{2-}) in lysimeter and drainpipe waters related to different nitrogen fertilization levels. The second one is to identify organic pollutants that potentially originate from used agrochemicals. The results of two-year investigating period (2014-2015) are presented in this study.

The experimental field was established in Potok near Popovača on distric Stagnosols. The research contained ten treatments with four replications where different nitrogen fertilizer levels were applied: 1. Control (no fertilization), 2. N_0 + P + K, 3. N_{100} + P + K, 4. N_{150} + P + K, 5. N_{200} + P + K, 6. N_{250} + P + K, 7. N_{250} + P + K + phosphogypsum, 8. N_{250} + P + K + zeolitic tuff + $CaCO_3$, 9. N_{300} + P + K, and 10. Black fallow – tillage only.

Phosphorous (P) and potassium (K) levels were constant, 120 and 180 kg/ha respectively. Each treatment area included two drainpipes (placed at the depth of 120 cm) and one lysimeter (at the depth of 80 cm) from which water samples were taken when discharge appeared. During the investigated period, 24 water samples for inorganic (13 from lysimeters and 11 from drainpipes) and 16 for organic substances analysis (six in 2014 and two in 2015 from lysimeters; five in 2014 and three in 2015 from drainpipes) were taken.

The concentrations of NO_3^- and SO_4^{2-} were determined by suppressed ion chromatography (Dionex ICS-1000 system) using IonPac AS17-C separation column fitted

with IonPac AG17 guard column. The gradient flow analysis was conducted using KOH as an eluent solution (10-30-10 mmol).

Ultra-high performance liquid chromatography (UHPLC) coupled to time-of-flight mass spectrometry (Q-TOF/MS) was used for non-target screening and quantification of organic substances. This allowed the search of the entire spectrum of various organic compounds and their quantification at high resolution (> 10000 FWHM, full width half maximal), with accurate mass accuracy (< 1 ppm) and satisfactory sensitivity in full-acquisition mode. Research results indicated that NO_3^- content in both lysimeters and drainpipes increased with rising doses of N fertilizer. In the investigated period the daily concentration varied from 0.52–265 mg/L in both lysimeters and drainpipes, depending on the treatment, precipitation and agrotechnical measures. Higher average concentrations for each treatment were observed in water samples from drainpipes (up to 126 mg/L), which are placed deeper in the soil (120 cm) confirming that nitrates do not bond with soil particles and are leached into groundwater. Daily SO_4^{2-} concentrations varied from 1-302 mg/L. As expected, the maximum average concentrations were recorded in treatment 7 with added phosphogypsum (184 mg/L in lysimeters and 105 mg/L in drainpipes), while the average concentrations on all other treatments were low (< 20 mg/L).

Non-target screening revealed more than 400 different organic substances (agrochemicals, hormones, opioids, antibiotics and various other drugs) in water samples. The correlation with applied plant protection products was not observed. The focus of this study was three herbicides: atrazine, simazine, and isoproturon. Atrazine and simazine have been banned in Croatia since 2004, while isoproturon is still used. The average annual concentrations of atrazine, simazine and isoproturon in water from lysimeters in 2014 were 1.40, 0.20 and 0.21 μg/L, respectively. In drainpipes the concentrations were 0.44, 0.20 and 0.04 μg/L, respectively. The average annual concentration of atrazine, simazine and isoproturon in lysimeters in 2015 was 4.69, 8.70 and 0.78 μg/L, respectively. In drainpipes the concentrations were 0.82, 3.82 and 1.66 μg/L, respectively. Due to the fact that atrazine and simazine have not been used in Croatia since 2004 and there have been no records of isoproturon use in the investigated area since 1997, it can be assumed that these compounds persist in the environment for a considerable length of time.

Keywords: ion chromatography, UHPCL Q-TOF/MS, groundwater quality

QUANTITATIVE ANALYSIS OF OVERLAPPED PEAKS

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One of the main goals in chromatography is to achieve optimum resolution in the minimum time. Often this is simply not possible, or it presents a time-consuming obstacle. Resolution values less than 1.5 between two partially separated peaks do not ensure accurate and precise quantitative analysis of components separated to a degree at which the area or height of each peak may be accurately measured. For that reason, we have developed a mathematical tool that can predict peak shape and area of potentially several unseparated peaks in such complex signals.

In our research we have used the Exponential Gaussian Hybrid (EGH) function to model the single peak. [1]

We have also tested our method on the following peak types that are frequently mentioned in the literature, the partial cases of the universal peak shape: Gaussian, Cauchy, Pseudo - Voigt (additive mixture of Gaussian and Cauchy), Fraser-Suzuki (asymmetric Gaussian), Laplace, asymmetric Laplace and asymmetric Cauchy. [2]

Because the measured signal is impaired by noise, some preprocessing is required. Denoised signal is then, using the Levenberg-Marquardt (LM) method, decomposed to a sum of EGH functions such that their superposition yields the processed signal with relative L^2 error ranging from 0.2% to 2%.

We have developed a universal and robust tool that can reconstruct complex chromatographic signal consisting of several unseparated chromatographic peaks.

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THE THROUGHPUT CHALLENGES IN ADME INVESTIGATION OF IBD DRUGS AND NUTRIENTS USING LIQUID CHROMATOGRAPHY AND *IN SILICO* METHODOLOGIES

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Crohn's disease and ulcerative colitis are two major forms of chronic inflammatory bowel disease (IBD). The etiology of inflammatory bowel disease is not fully clarified, but it is known to be an immune disorder in genetically predisposed individuals. Incidence of IBD is increasing worldwide, especially in developing countries and Asia. IBD has a peak incidence in both men and women aged between 20 and 35. Therefore, IBD usually affects women during their reproductive years and many concerns arise among these young patients. Treating IBD in pregnant women presents unique challenges. The key to a successful pregnancy of IBD patients is achieving and maintaining disease remission. If necessary, the safest drug possible should be used, with attention to the optimal efficacy for the patient's condition. Regarding folic acid, special attention is given to the increased risk of malabsorption, as well as the effects of some drugs, such as sulfasalazine, on folate metabolism. The 2010 European Crohn's and Colitis Organisation guidelines state that all IBD patients should be prescribed 1 mg folic acid daily in anticipation of pregnancy. Moreover, several studies have shown that some patients need to be on even higher doses of folic acid than the standard dose (5 mg/day). The fixed-dose combinations of IBD drugs and folic acid are not available on market. To give insight into compatibility of active pharmaceutical ingredients (APIs) in proposed fixed-dose combinations, investigation of their ADME properties is of exceptional importance [1,2].

In this study ADME data of selected APIs obtained by two different high-throughput tools were compared. *In silico* ADME studies use various models developed for predicting ADME properties of compounds based on their chemical structures. Afterwards these properties are used to simulate pharmacokinetics of novel compounds. Sophisticated chromatographic columns obtained by immobilization of phospholipids and plasma proteins onto the surface of silica particles make liquid chromatography an attractive high-throughput technique in ADME investigation.

ADME properties of IBD drugs and folic acid were predicted by 15 *on-line* available computer platforms. Furthermore, absorption and plasma protein binding of selected APIs were investigated by C18-HPLC, IAM-HPLC and HSA-HPLC. The logk_w values obtained by C18-HPLC were in the range from 0.45 (6-mercaptopurine) to 1.34 (azathioprine), while logk_w values obtained by IAM-HPLC were lower, from -0.37 (folic acid) to 0.68 (azathioprine). To evaluate plasma protein binding of APIs by HSA-HPLC technique the method was validated using literature plasma protein binding data [3] (r=0.98). The lowest plasma protein binding was found for 6-mercaptopurine (20%), while the folic acid showed the longest retention time on the HSA-HPLC column and subsequently the highest plasma protein binding (69%) among the investigated compounds.

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Acknowledgment

This work has been supported in part by the Croatian Science Foundation under the project number [UIP-2017-05-3949].

SIMPLE AND FAST HPLC-DAD METHOD FOR DETERMINATION OF HCDC ACTIVITY AND FORMATION OF VINYLPHENOL IN SACCHAROMYCES AND NON-SACCHAROMYCES YEAST

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Conventionally, alcoholic fermentation in the production of wine is performed by yeast species *Saccharomyces cerevisiae*. There are numerous starters available, however due to the growing demand for wines with specific characteristics, other Saccharomyces and non-Saccharomyces species are being investigated for potential use as starters [1]. Yeast selection has involved the development of techniques for detecting strains that might improve wines in terms of aroma, structure, colour and other technological properties [2]. Colour of the wine can be affected as some metabolites produced by yeast during fermentation may react with grape anthocyanins to produce highly stable pyranoanthocyanins. For the facilitation of formation of vinylphenolic pyranoanthocyanins, yeast strains with high hydroxycinnamate decarboxylase activity are used (HCDC). The mechanism of reaction is decarboxylation of hydroxycinnamic acids and formation of vinylphenols that condense with grape anthocyanins and form stabile vinyphenolic pyranoanthocyanin adducts [3]. It has been demonstrated that some non-Saccharomyces strains (*Pichia guillermondii*, *Schizosaccharomyces pombe*) have positive HCDC activity and they can produce vinylphenolic pyranoanthocyanins in higher concentrations than *S. cerevisiae*. A simple way of determining whether the yeast strain has HCDC activity or not, is the use of fermentation media with the addition of hydroxycinnamic acids, such as *p*-coumaric acid. The degradation of *p*-coumaric acid and transformation into 4-vinylphenol (and possibly in 4-ethylphenol) can be checked by LC-DAD. Most of the published data has been done on smaller number of strains.

The goal of our work was to develop simple method for the screening of Slovenian in-house yeast collection, comprising of native isolates that mostly originated from Vipava valley and Karst region, and therefore try to determine strains with high HCDC activity. These strains can be used for wine fermentations in order to produce more stable pyranoanthocyanins; which

is especially important in wines that has less anthocyanin concentration already from the grape, such as Pinot Noir.

103 different yeast strains belonging to 28 species were selected for the assessment of HCDC activity. In some cases the difference in *p*-coumaric acid metabolism rate between two strains exceeded 90%. All tested *S. paradoxus* strains showed higher than 40% degradation rate of *p*-coumaric acid. HCDC activity of *S. cerevisiae* strains which is the species most commonly used in fermentation, varied between 5.1 and 66.1%. In the commercial strains tested, FPC and EC118 showed 43.9 and 21.5% conversion rate, respectively. It was observed that some native strains had higher HCDC activity than commercial ones. Three strains produced vinylphenol in concentration higher than 50 ppm, two of them being *P. guillermondii* and another strain being *S. paradoxus* (Sut85). In general strain with high HCDC activity also produced high concentration of 4-vinylphenol.

The results showed that HCDC activity is highly strain dependent, which correlates with the literature data available. The proposed method is very simple and does not require special sample preparation prior to HPLC analysis. Furthermore, the proposed fermentations in deep-well microtiter plates allow the screening of high number of strains. The method could be used for routine screening, to determine which strain has high HCDC activity and produces high concentration of vinylphenols and can therefore be used in future for determination of strains ability to synthesize vinylphenolic pyranoanthocyanins.

Key words: yeast, hydroxycinnamate decarboxylase, 4-vinylphenol

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DETERMINATION OF AFLATOXIN M1 IN MILK USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Growth of mold on cereal grains may result in the formation of secondary metabolites – mycotoxins. [1] Mycotoxins may occur during any stage of production and transformation into a food product. They can be produced in plants infected in the field, during harvesting, storage and transport, technological transformation and food preparation. [2]

Aflatoxins are one of the most significant and most serious causes of mycotoxicosis in humans and animals. [1] They are primarily produced by two species of *Aspergillus fungus* (*A. flavus* and *A. parasiticus*). *A. flavus* produces B aflatoxins and *A. parasiticus* produces B and G aflatoxins. [3] Aflatoxins are extremely toxic, mutagenic, teratogenic and carcinogenic compounds. Aflatoxin B1 (AFB1) is the most carcinogen aflatoxin. [2] Aflatoxin M1 (AFM1) is a hydroxylated product of AFB1 and it is found in animal and human breast milk. [1] AFM1 is a relatively stable compound in raw and processed milk. Since it can't be eliminated by pasteurization it represents a serious health concern. [2]

In this study the method for determination AFM1 in milk samples has been validated according to the Commission Decision 2002/657/EC. The samples were defatted and passed through the immunoaffinity columns (IAC) VICAM Afla M1^{TM} HPLC. Acetonitrile was used for elution. After evaporation the samples were reconstituted in ultrapure water and acetonitrile, and analyzed by the HPLC-MS/MS.

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SEPARATION / ISOLATION / STRUCTURE ELUCIDATION VIA LC-SPE/NMR

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Ultra-high performance liquid chromatography (UHPLC) was employed for initial analysis of the 3-bromo-5-(trifluoromethyl)aniline samples in order to detect and quantify individual process impurities. 3-bromo-5-(trifluoromethyl)aniline is a starting material used in the synthesis of active pharmaceutical ingredients (APIs). Hyphenated system of liquid chromatography, solid phase extraction and NMR spectroscopy with cryoprobe (LC-SPE/NMR) was used to evaluate the applicability of the modern approach to isolation and identification of low levels of impurities. It was proved to be efficient for separation, isolation and trapping of more than 15 3-bromo-5-(trifluoromethyl)aniline related analytes on SPE cartridges. Based on the analysis of one-dimensional ^{1}H and ^{19}F and two-dimensional homo- and heteronuclear NMR spectra (COSY, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC) and evaluation of MS results the structures of several isolated impurities were proposed.

OLIGOSACCHARIDE ANALYSIS BY LIQUID CHROMATOGRAPHY

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Oligosaccharide analysis has always been a challenge due to oligosaccharide complexity; diversity of monosaccharide unites (often with the same mass but with different charge and hydrophilicity) combined with a multiplicity of ways in which they can be linked together, can create the wealth of oligosaccharide structures. Nowadays, different liquid chromatography techniques are used for oligosaccharide analysis with high-performance liquid chromatography (ranging from normal phase and hydrophilic interaction chromatography, to ion-exchange and reversed-phase chromatography) and ultra-high-performance liquid chromatography (often reversed-phase and hydrophilic interaction chromatography) being the most common ones. Each of these liquid chromatography types has its own advantages and flaws in oligosaccharide analysis, and often for complete understanding of an oligosaccharide structure there is a need for combination of different chromatography techniques as well as combination of liquid chromatography with mass spectrometry. Therefore, an analyst/researcher should carefully consider which technique is the most appropriate for a specific problem and which information can be obtained /missed by each of this technique.

MULTI-DIMENSIONAL LIQUID CHROMATOGRAPHY COUPLED WITH MASS DETECTION

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THE INFLUENCE OF THE LAB WATER QUALITY ON LIQUID CHROMATOGRAPHY

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HPLC is one of the most widely used analytical techniques in the laboratory. HPLC is applicable in pharmaceuticals, food and beverage industry, scientific research, clinical trials, *etc*. HPLC techniques have been advancing greatly in recent years in their accuracy, speed, repeatability and reliability.

To maximize the benefits of all these advantages of HPLC techniques, it is most important to have water, a good laboratory water, which is the most commonly used reagent in all kind of laboratories.

Consequently, systems and techniques for water purification have been developed. The two main purposes of the water system are: (i) to prevent interference with the sample and (ii) optimization of the performance of the analytical method of HPLC. This can be achieved by removing impurities in laboratory water.

DETERMINATION OF 31 SUSPECTED FRAGRANCE ALLERGENS BY GC-MS IN VARIOUS COSMETIC PRODUCTS USING MASS SPECTRUM LIBRARY AS CONFIRMATION TECHNIQUE

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Manufacturers of cosmetics were required, by the European Cosmetics Directive (2003/15/EC) published in 2003., to indicate 26 fragrance substances identified as potential allergens in cosmetic products if they exceed a threshold of 0.01 % for rinse-off and 0.001 % for leave-on products.

Cosmetic products are very complex, containing sterols, waxes, flavonoids and preservatives. Since fragrance components contain compounds having similar structures and small molecular mass it is very difficult to determine, identify them and not to get false positive results.

The present method describe determination of 31 (α-amylcinnamaldehyde, cinnamal, citral, hydroxycitronellal, α-hexylcinnamaldehyde, lilial, lyral, phenylacetaldehyde, α-amylcinnamic alcohol, benzyl alcohol, cinnamyl alcohol, citronellol, eugenol, farnesol, geraniol, isoeugenol, linalool, anisyl alcohol, methyl eugenol, 4-allylanisole, benzyl benzoate, benzyl cinnamate, benzyl salicylate, camphor, 1,8-cineole, coumarin, d-limonene, *iso*-α-methylionone, methyl 2-nonynoate, methyl 2-octynoate, safrole) suspected fragrance allergens by Shimadzu 8050 GC-MS/MS.

Fragrance components in cosmetic products (gel for oily skin, cream for dry skin, shower gel, toothpaste) are extracted with methyl tert-butyl-ether (MTBE) and injected to GC-MS/MS. Their identification and quantification is achieved through selected ion monitoring (SIM; SIM-SCAN; SCAN). The capillary column was DB-17MS (20 m × 0.18 m × 0.18 μm). For all the fragrance compounds it was achieved excellent peak resolution and good match with the mass spectrum from the library.

Calibration curve was made using standard solutions in concentration from 0.1 mg/L to 1 mg/L and internal standards 1,4-dibromobenzene and 4,4-dibromobiphenyl in concentration of 0.6 mg/L. Limit of quantification of method is 0.0001 % and recovery was at the range from 75 to 110 %.

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PROFILING OF THE PROTEINS IN HUMAN TISSUES AND BIOFLUIDS

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Human tissues and biofluids are important source of molecular information for determination of the patient status. Presence and expression of proteins in human tissues and biofluids can give valuable insight into disease activity, therapy response and disease progression. Human tissues are unique and precious and therefore limited in number. Existing protocols for tissue analysis are typically involving large tissue pieces while their translation for minute samples is difficult. Moreover, while detection of the disease-related compounds in human tissues is of special importance, their presence in biofluids makes them more available due to minimally invasive sampling opportunities. To address this issues, in our research we focused on development of analytical methodologies for detection of proteins in small tissue sizes. Moreover, we developed microfluidic device to facilitate protein digestion using immobilized enzyme reactor (IMER).

Sample preparation and long digestion times prior to LC-MS analysis is often considered bottleneck for high-throughput analysis. Following procedures used in pathology we investigated influence of sample preparation on subsequent proteome analysis. We evaluated several methods to measure the proteome of the human tissues ranging from 1-2

mm2 at thicknesses of 10, 16 and 20 μm. Overall methods showed good reproducibility and allowed detection of small changes in protein abundance across the tissues. Results showed variations in proteomes with sample treatment and that method using SDS resulted in less identified proteins as compared to ACN/urea only. However, quantitation of proteins in the samples showed more efficient extraction of proteins by SDS. Increasing tissue thickness resulted in higher number of identified proteins and corresponding quantitative values.

Protein conversion into the peptides via endoproteinases is one of the most important steps in bottom-up proteomics and 18 hours is mostly used for proteins digestion. To speed up proteins digestion we developed cyclic-olefin-copolymer IMER which allowed shorter digestion in order of seconds or minutes. In the designed IMER, trypsin was immobilized on polymer monolith via vinyl azlactone chemistry and IMER was evaluated on model proteins and dried-blood-spots (DBS). Results showed that IMER was fast and effective for offline digestion of model proteins and human DBS and IMER-facilitated digestion resulted in similar performance of sequence coverage and peptide numbers for model proteins as compared to in-solution digestion. While both methodologies showed comparable number of total proteins identified, IMER method considerably shortened the total workflow.

ION CHROMATOGRAPHY SYSTEM FOR COMPLETE ANALYSIS OF FRESH WATER AND WASTEWATER

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Ion chromatography (IC) is an established technique worldwide for monitoring water, including surface, ground, drinking, and wastewaters. To determine water purification system effectiveness, common inorganic anions, alkali and alkaline-earth cations, and transition and heavy metals are monitored and determined by IC. Incoming wastewater (influent) is monitored to assess the conditions of the feed water. Similarly, outgoing (effluent) wastewater is monitored prior to discharging into the water system to prevent any adverse environmental effects and comply with discharge permits.

Calcium and magnesium are routinely measured to determine water hardness, an important parameter for corrosion control. Ammonia is a colorless, pungent gas. It is highly soluble in water in ionized form (the ammonium cation, NH_4^+). The extent of its toxicity to aquatic life depends upon the extent of dissociation, which in turn depends upon temperature and pH. Ammonia can enter environmental waters as a product of anaerobic decomposition of nitrogen-containing compounds or from waste streams containing ammonia.

Inorganic anions, including fluoride, nitrite, and nitrate are considered as contaminants and are monitored in water. High levels of fluoride cause skeletal and dental fluorosis, and nitrite and nitrate can cause methemoglobulinemia, which can be fatal to infants. Other common anions, such as chloride and sulfate, are considered as secondary contaminants. By IC, it is also possible to determine total phosphorus and total nitrogen (inorganic and organic phosphorous and nitrogen).

For transition metals analysis, IC has several advantages including the ability to analyse oxidation state speciations (*i.e.* Fe^{2+}/Fe^{3+}, Cr^{3+}/Cr^{6+}, Sn^{2+}/Sn^{4+}, etc.) with the multi-element analysis in a single run. Contrary to the low concentration of metals, the relatively high concentrations of alkali and alkaline-earth metals in environmental samples represent a

limitation in use of IC for direct metal analysis. In order to deal with this challenge, significant work has been done utilizing matrix elimination and/or pre-concentration in order to minimize interference and achieve lower detection limits.

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MODERN METHODS OF SAMPLE PREPARATION FOR CHROMATOGRAPHIC ANALYSIS OF CONTAMINANTS IN FOOD – PRACTICAL EXAMPLES

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Pesticides, mycotoxins, heavy metals, polycyclic aromatic hydrocarbons, dioxins, PCB, histamine, melamine, acrylamide and other contaminants are regularly monitored in food in the EU. Methods for the determination of contaminants in food must be capable to determine very low concentrations of analytes (e.g. 0.01 μg/kg of aflatoxin M1 in milk). Apart from low concentration of analyte, the large interference problem is caused by very complex matrices. In order to achieve high sensitivity and selectivity, various techniques for sample preparation with modern instrumental determination methods are combined. Over the years, new, modern techniques for sample preparation have been developed. The lecture will present several practical examples that combine modern methods of sample preparation with chromatographic detection methods.

MICRO-HPLC-UV ANALYSIS OF COCAINE AND ITS ADULTERANTS IN ILLICIT COCAINE SAMPLES SEIZED BY AUSTRIAN POLICE FROM 2012 TO 2017

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The worldwide consumption of illicit drugs presents a big problem in terms of health care and prosecution. In the recent years, hundreds of novel psychoactive substances came up and were traded via the Internet, but there is still a big demand for classic illicit drugs such as cocaine, heroin, cannabis and ecstasy. Among these, cocaine particularly is frequently altered not only with excipients, but also with other physiologically active substances.

The goal of this work was to develop and validate a simple micro-HPLC method with UV detection of cocaine and its adulterants at four different wavelengths. The advantages of this approach were decreased costs of analyses in comparison with usual LC-MS/MS analyses and minimized production of organic waste due to micro flow rate of mobile phase which was 70 μL/min.

Six cocaine adulterants were chosen according to the estimation of their appearance frequency in cocaine samples seized by the Austrian police: caffeine, procaine, levamisole, phenacetin, lidocaine and benzocaine. The analytes were separated on the C18 column. The detection wavelengths of the analytes were set to maxima of the UV absorbance spectra to improve the sensitivity and selectivity. Adequate pH value of the mobile phase was estimated to range from 4.5 to 6.0 according to pKa values of analytes with respect to the optimal pH range for C18 column operation and pH of 5.5 was proven to give best results in terms of selectivity and peak shapes. Application of gradient elution improved peak shapes of strongly retained compounds and time of analysis was set to 18 minutes. Sample concentration was adjusted to 0.5 mg/mL and injection volume was 0.1 μL to ensure sample solubility in given diluent, achieve optimal sensitivity, and to prevent column overload and analyte carry over.

After development and validation, the method was applied for the analysis of 110 cocaine samples seized from dealers in Austria to reveal the trend in terms of composition of the samples from 2013-2017. Cocaine was found as its hydrochloride salt in all cases and the purity grades varied considerably. Purity of the cocaine HCl increased during the last years. The most frequent adulterants were levamisole HCl and phenacetin, followed by caffeine and lidocaine HCl. There was a trend of higher purity of cocaine samples while number of adulterants became less in term of mass fraction and frequency of appearance. The variety of composition might be additionally dangerous for consumers, who cannot estimate the proper amount for consummation.

Acknowledgments

The work was carried out under cooperation in the frame of CEEPUS, project CIII-RO-0010-10-1516 and CIII-RO-0010-10-1617.

This work is published in Journal of Liquid Chromatography and related technologies:

1. K. Vinković, N. Galić, M. Schmid, *J. Liq. Chromatogr. Rel. Technol*. **41** (2018) 6–13.

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