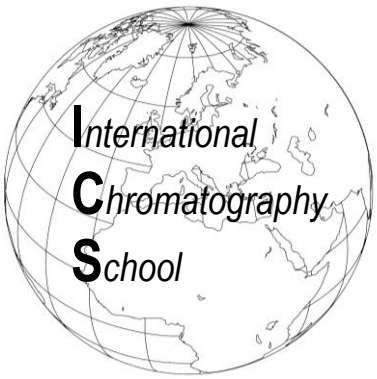
UNIVERSITY OF ZAGREB

FACULTY OF CHEMICAL ENGINEERING AND TECHNOLOGY

16th International Chromatography School



BOOK OF ABSTRACTS

9th – 10th June 2016

ZAGREB, CROATIA

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PREFACE

Welcome to 16th 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 and sponsors for their contributions at 16th ICS.

LECTURES

DETECTING COMPOUNDS IN NATURAL PRODUCTS WITH UHPLC COUPLED WITH CHARGED AEROSOL DETECTION

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This presentation will discuss the latest chromatographic approaches to characterize Traditional Chinese Medicines (TCM), Ayurvedic medicines, botanical preparations, and herbal supplements. State-of-the-art UHPLC separations coupled with charged aerosol detection (CAD) are being used to profile and measure key analytes in these complex products. Analytical throughput and analyte resolution can be markedly improved using the new Thermo Scientific™ Vanquish™ UHPLC system.

Botanicals contain a great diversity of compounds that exhibit wide variation in their physicochemical properties. Although no single analytical method is available to measure all potentially active components, HPLC with charged aerosol detection is a nearly universal approach that nonselectively measures any nonvolatile and many semivolatile compounds; that is, CAD does not require that analytes be ionizable (as required for mass spectrometry) or contain a chromophore (as required for UV spectrophotometry).

Presented are several HPLC/UHPLC methods with charged aerosol detection that have been improved to increase speed and sensitivity. The improved methods were evaluated for the measurement of phytochemicals extracted from a variety of botanical and herbal supplements including: triterpene glycosides from black cohosh (*Cimicifuga racemosa*) and *Bacopa monnieri*; oxypregnane glycosides from *Caralluma fimbriata*; flavonolignans from milk thistle (*Silybum marianum*); steroidal lactones from Ashwagandha (*Withania somnifera*teroidal and oxypregnane glycosides from *Hoodia gordonii*.

VIRTUAL COLUMN - SEPARATION SIMULATION TOOL IN ION CHROMATOGRAPHY

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How it is possible to develop method and run chromatogram in few minutes? That is main goal of this presentation. We are going to show easy method development with many possibilities in interaction whit audients for few cations, anions and carbohydrates. From predicted to real chromatogram – differences.

Virtual column is a simulation tool that uses known ion chromatographic retention data to predict new retention data and chromatograms. The known retention data was acquired by Dionex using an appropriate experimental design and then embedded into Virtual Column.

DETERMINATION OF ACRYLAMIDE IN FOOD BY ION EXCLUSION LIQUID CHROMATOGRAPHY AND DIODE ARRAY DETECTION

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On 4 June 2015, European Food Safety Authority (EFSA) published its first full risk assessment of acrylamide in food. Experts from EFSA's Panel on Contaminants in the Food Chain (CONTAM) reconfirmed previous evaluations that acrylamide in food potentially increases the risk of developing cancer for consumers in all age groups.

Acrylamide is a chemical that is naturally formed in starchy food products during high-temperature cooking, including frying, baking, roasting and also industrial processing, at +120 °C and low moisture. The main chemical process that causes this is known as the Maillard Reaction; it is the same reaction that 'browns' food and affects its taste. Acrylamide is formed from sugars and amino acids (mainly one called asparagine) that are naturally present in many foods. It is found in products such as potato crisps, French fries, bread, biscuits and coffee. It was first detected in foods in April 2002 although it is likely that it has been present in food since cooking began. Acrylamide also has many non-food industrial uses and is present in tobacco smoke [1].

A simple and inexpensive method for determination of low concentration of acrylamide in food (bread, French fries and biscuit) was developed and validated using ion exclusion liquid chromatography with diode array detection. The method is based on separation of acrylamide on ion exclusion column Aminex HPX-87H using 0.01 mol/dm^3 sulfuric acid as mobile phase flowing under isocratic elution. The flow rate was 0.6 mL/min with the run time of 55 minutes. Detection is performed at 200 nm. Calibration curve was made using standard addition method by adding known amounts of acrylamide (35, 89, 178, 355, 711, 1422, and 3554 μg/L) to the sample which doesn't contain acrylamide. Better results have been achieved taking into account matrix effect, and there is no need to use internal standard. This matrix effect has been taken into account when plotting regression lines because there were significant differences between the slope determined in pure water and that obtained by the standard addition method. Value of the slope in the pure water was 1910 and value of the slope in the matrix was 1017.

The extraction of acrylamide was done with ultrapure water and samples were left to stand in a termostated water bath heated at 70 °C for 30 minutes. After centrifugation, samples were filtered and analyzed on HPLC.

Validation of method for biscuit sample was done using FAPAS certified reference material (T3051QC) with content of acrylamide in concentration of 227 μg/kg. For other samples (bread and French fries) standard addition method was used for the validation of method. Limit of quantification of method was 60 μg/kg. For all of the samples recoveries were from 85 to 120 %. Expanded uncertainties of measurements were from 27 to 32 %.

The content of acrylamide was 227 μg/kg for biscuit, 120 μg/kg for bread, and 330 μg/kg for French fries. For now, there is only indicative acrylamide values in food based on the EFSA monitoring data from December 2007.

References:

1. Official Journal of the European Union 2013/647/EU
2. Journal of Chromatography A, 1077 (2005) 128-135

CHROMATOGRAPHY SEPARATION AND DETECTION OF NITRATE IONS IN LETTUCE (*Lactuca sativa*)

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Lettuce (*Lactuca sativa*) is an annual plant of the daisy family Asteraceae. It is most often grown as a leaf vegetable. It can accumulate higher amount of nitrate that may harm the health of the consumer as it can be converted to nitrite causing methaemoglobinaemia or carcinogenic nitrosamines. Depending on growing conditions and harvesting time of lettuce NN (146/12) prescribed maximum level of nitrates is 3000 mg NO_3^-/kg in fresh plant material. According to Statistical Yearbook of the Republic of Croatia average annual production of lettuce in period from 2000–2014 was 3.64 t/ha and it was in the range form 1.77 t/ha in 2014 up to 6.54 t/ha in 2007.

In order to determine the effect of different nitrogen [KAN N(MgO) 27% (4.8%)] fertilization levels on the accumulation of nitrate content in lettuce field trial was set up at Miroševac location in Zagreb. The trial involved a monofactorial field experiment with five increasing N-fertilization levels (0, 50, 100, 150, 200 kg N ha^{-1}) in randomized block design with five replication (1 m^2 plot area for each replication). In each plot twelve plants were planted in April 2014. Sampling of plan material was conducted in July 2014. From each plot four randomly selected heads of lettuce were taken. Samples were dried at 70 °C for 72 h and weighed. Nitrate concentration was measured by extracting 30 mg of grounded dry plant material in 30 mL of deionized water at 90 °C for 2 h. 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. Nitrate separation and detection in extract was performed on Dionex ICS-1000 system: separator column [Ion Pac AS 17 (4×250 mm) Dionex]. Eluens concentration (KOH) was changed by eluens generator module (EGC-KOH, RFC-30) with on-line eluens preparation: the concentration was 10 mmol/L for the first 5.5 minutes and then was increased to 35 mmol/L, which was reached in 16.5 minutes. Total time

of anions analysis was 18 minutes. After 18 minutes all seven anions left the column and were detected on a detector, but due to simultaneous analysis of cations, which takes 30 minutes, for the last 12 minutes the concentration of anion eluens was 10 mmol/L. Flow rate was 1.0 mL min^{-1} and for detection suppressed conductivity was used.

Observed data were subjected to 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 excessive mineral nitrogen fertilization did not significantly effect on enhancement of accumulated nitrates (NO_3^-) in the lettuce leaf. Average values in fresh plant material were in the range from 697.9 mg NO_3^-/kg at treatment with applied 50 kg N/ha to 878.9 mg NO_3^-/kg at treatment with applied 100 kg N/ha. It is important to emphasize that the determined values do not exceed the maximum levels prescribed by NN (146/12).

Additionally, contents of fluoride, chloride, sodium, potassium, ammonium, magnesium and calcium were also separate and detected by means of ion chromatography system and average values, for all five treatments in fresh plant material, were: 5.3 g F^-/kg; 36.4 g Cl^-/kg; 13.5 g Na^+/kg; 155.3 g K^+/kg; 18.9 g N-NH_4^+/kg; 10.7 g Mg^{2+}/kg and 13.1 g Ca^{2+}/kg.

Key words: Chromatography Separation, Mineral Fertilization; Lettuce

DETERMINATION AND VALIDATION OF FREE CYANIDE IN WATER BY ION CROMATOGRAPHY

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Cyanide is one of the most toxic inorganic substances. It is emitted into the environment mainly with industrial waste water. Its presence in concentrations higher than allowed one is a potential threat to the human health. Cyanide is classified according to its availability in the presence of complexing metals. Total cyanide refers to cyanide which can be released as hydrocyanic acid from the aqueous sample under total reflux distillation conditions and includes both free and total complex cyanides. Free cyanide refers to cyanide which can be released as hydrocyanic acid from the aqueous portion of a sample by direct cyanide determination without reflux distillation. In Croatia, the maximum allowed value for free cyanide in waste water is up to 100 µg/L, for total cyanide up to 500 µg/L, in water for human consumption maximum level for total cyanide is 50 µg/L. A number of procedures for the determination of cyanide have been proposed (titration, colorimetric and polarographic techniques, ion-selective electrode).

In this paper, analysis of free cyanide in waters wass carried out by using ion chromatography (Compact IC, Metrohm AG, Switzerland) with electrochemical detector (amperometry), working electrode (Ag), and reference electrode Ag/AgCl. Used column was Metrosep A Supp 1 (250 × 4.6 mm) and the corresponding pre-column was commercially available from Metrohm. The column packing was polystyrene/divinylbenzene copolymer, particle size 7 µm. The volume injected, the flow rate and the temperature were set at 20 µL, 1.0 mL/min and 25 °C, respectively. The eluent was 100 mM NaOH (pH 12.8). Retention time of cyanide was 6.5 min. Water samples should be stabilized immediately with sodium hydroxide (adjust pH above 12), cooled to 4 °C, before injection filtered through a membrane filter pore size 0.45 µm.

The method was validated; validation parameters were studied in terms of linearity, precision, trueness, LOD and LOQ. The linearity of cyanide response was determined by measuring three replicates of seven standards (1, 5, 10, 50, 75, 100, 200 µg/L). The

calibration results showed good linearity ($r > 0.999$). RSD of repeatability was below 1 % and RSD of inter-day precision for standard was below 3 %. Mean recoveries from 100.7–104.5% were obtained for samples spiked with cyanide over the range of 45–125 μg/L. Limits of detection and quantification were 0.5 and 1.4 μg/L, respectively.

Method could also be applied for determination of total cyanide but with appropriate modification to the standard reflux-distillation procedures.

WHAT IS HIGH PRESSURE ION CHROMATOGRAPHY? WHY DEVELOP HIGH PRESSURE ION CHROMATOGRAPHY CAPABILITIES?

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High-pressure ion chromatography systems enable continuous operation at system pressures up to 5000 psi, making it possible to use new, smaller 4 µm particle-size ion-exchange columns in capillary and analytical scale formats.

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High backpressure tolerance is intended to let users increase flow rates to maximize throughput while still providing the advantages of electrolytic eluent.

GC×GC-TOF MS - FAR BEYOND A ROUTINE TARGETED SCREENING LEVEL IN ENVIRONMENTAL AND FOOD SAFETY ANALYSIS

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Although the basics of Time of flight mass spectrometers (TOF MS) were established in 30's of 20th century, due to the lack of fast electronic the renewal of this technology was postponed to early 90's of last century. At this time the enormously fast, but extremely sensitive, first commercially available GC-TOF MS were introduced to the market. The nature of TOF MS technology, such as fast and sensitive acquisition of unskewed MS spectra, was followed by implementation advanced mathematical algorithms of data mining – automated peak find, deconvolution and scripting.

The potential of fast GC-TOF MS instruments equipped with ion source not requiring its cleaning was later on extended by their combination with comprehensive gas chromatography (GC×GC).

The increased nowadays demands for analytical instrumentation can be summarized as: "One run covering all analyst's requirements, such as target and non-target screening, qualitative and quantitative capabilities along with easy and fast hardware-software handling and reasonable data file sizes". Such requirements were the leading idea in a development of the high-resolution instruments (*e.g.* employing the multi-reflection TOF MS analyzer so called Flight Folded Path, FFP™). The ultimate mass resolution (>50.000 FWHM) and mass accuracy (<1 ppm) along with the ability to hyphenate it with GC×GC option extending its capabilities to the edge of possible – significantly enhanced separation, identification and confirmation potential, as will be demonstrated in this contribution on case studies dealing with different type of analyses and sample examinations.

DEVELOPMENT OF HS-SPME GAS CHROMATOGRAPHY-MASS SPECTROMETRY METHOD FOR AROMA COMPOUNDS ANALYSIS IN FOOD SAMPLES

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The characteristic flavors of food and beverage products are generally the result of complex multisubstance mixtures, containing several hundred compounds characterized by different chemical structures and physicochemical properties. These mixtures comprise a wide range of organic chemical entities. They are characterized by different polarities and volatilities; occur in trace concentrations (ppm to ppt range) in sample. However, their presence decisively influences to the characteristic aroma profiles.

The analysis of naturally volatile and semivolatile organic aroma compounds is generally performed by implementing gas chromatographic (GC) methods. Qualitative analysis and identification of the molecules that give the distinctive odor or taste to foods can be confirmed by mass spectrometry (MS). Sample preparation of aroma compounds often requires extraction and concentration before gas chromatographic – mass spectrometric analysis (GC-MS). Solid phase microextraction (SPME) is a simple, solventless extraction procedure in which a phase-coated fused-silica fiber is exposed to the headspace (HS) above a liquid or solid sample.

Developing HS-SPME GC-MS method for food samples is time consuming. The method must be optimized by varying different parameters such as fiber type, extraction temperature, equilibration, extraction and desorption times, sample volume, effect of salt addition, stirring velocities and sample weight. In addition, it is necessary to choose the right GC column and GC inlet liner to obtain optimized separation and better peak shape needed for quantification of a certain analyte. Although, there are many literature reviews describing HS-SPME GC-MS method development, practical experience in method developing is crucial factor to achieve method with a good reproducibility and sensitivity for analysis of different samples.

TWO-DIMENSIONAL LIQUID CHROMATOGRAPHY (2D-LC) – EMPHASIS ON RESOLVING COMMON CHROMATOGRAPHIC ISSUES

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Routine liquid chromatographic analysis is facing with four major challenges:

1. Selectivity
2. Sensitivity
3. Assay robustness
4. MS compatibility

Complex samples, especially those from biological sources, have challenging matrices, often with coeluting peaks.

Regulatory institutions require detection at ppb or even ppt levels. Therefore, tight control over potentially toxic materials that are present in products that we consume on daily basis is mandatory.

It is difficult to set up a universal sample preparation method to determine same analytes in diverse samples.

And finally, most of LC-UV methods end up as inlet methods for MS analysis as analysts need to verify and prove identities of unknown compounds.

All those challenges can be simplified and resolved using generic 2D-LC systems that offer a setup of an advanced chromatographic tool and solve even the toughest analytical problems without a need for complex instrumentation.

IMPORTANCE OF SAMPLE PREPARATION FOR CHROMATOGRAPHIC ANALYSIS

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Sample preparation is a key step in every analytical workflow. It requires a lot of extensive research, and takes a long time to develop. Current markets offer a multitude of consumables and automated platforms to enable fast, reproducible and easy sample preparation whatever workflow requirements may be. This work compares Soxhlet, which is the most commonly used method of extraction in laboratories today, with accelerated solvent extraction. Important factors of sample preparation are the homogenization of the sample, increasing/decreasing analyte concentration, removing interfering chemicals, liberation of the analyte from the sample matrix, modifying chemical structure, or changing sample phase. Accelerated solvent extraction (ASE) is a method of extraction that reduces the extraction time of a single sample to 12 – 20 minutes. The sample can be cleaned in-line, which cuts the costs of preparation, solvents and saves time. Accelerated solvent extraction enables unattended extraction of up to 24 samples. This work presents ready to use applications for accelerated solvent extractions, and several studies that compare recovery of analyte using Soxhlet extraction and ASE. Various studies that compare chromatograms of identical samples obtained using different sample preparation methods, such as: Soxhlet and Acid digestion, are also presented in this work. ASE is nowadays a certified method for extraction of pesticides (OCP, OPP), herbicides, semivolatiles (BNAs) and PCBs from soli, sediments and sludges. It also meets the requirements of U.S. EPA Method 3545 for extraction of polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans from environmental samples.

HPLC DETERMINATION OF MYCOTOXINES IN FOOD

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Mycotoxins are poisonous chemical compounds produced by certain fungi. There are many such compounds, but only a few of them are regularly found in food and animal feedstuffs such as grains, seeds, and dried fruit. Aflatoxins are mycotoxins that are produced by various Aspergillus Flavus molds. Not only that are these compounds extremely toxic, but they are also mutagenic, teratogenic and carcinogenic.

This method describes HPLC determination of aflatoxins B1, B2, G1 and G2 in food with immunoaffinity column clean up and post-column derivatization according to official method HRN EN 14123:2008.

HSS-GC-FID DETERMINATION OF ORGANIC VOLATILE COMPOUNDS AS POTENTIAL MARKERS FOR QUALITY OF OLIVE OIL

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Olive oil is a product of great value due to its nutritional and pharmaceutical qualities. That it is so beneficial is known from ancient times. It is the main source of fat in the Mediterranean diet, but in the recent years that olive oil is beneficial is known and its use in diet is recommended worldwide. There are many research conducted on the olive oil's protective effect against cardiovascular disease, anti-inflammatory effects, but also possible use in cancer prevention and in Alzheimer patients.

It is obtained from the olive drupes by different types of extraction which has several phases, one of the most important ones being crushing. Today a lot of attention is given to the use of Virgin olive oils, which are obtained solely by mechanical process or another physical process. A certain thermal conditions that do not lead to any alterations are required during the process. The olive drupes and the oil undergo only washing, decantation, centrifugation and filtration. Additives of any kind are not permitted. Extra virgin oil has a distinctive taste and it is rich with phenolic antioxidants, the main components that contribute to powerful health benefits of olive oil. Unfortunately, it is comment that lower quality olive oils have been processed and adulterated with cheap oils.

The importance of the olive oil, as well as the increasing demands of the consumers and the price of this product requests a good quality. The European commission and the International Olive Oil Council have agreed on a set of parameters and particular tests for checking the olive oil quality. Some of them are the tests for olive oil free acidity, UV extinction test, Folin-Ciocalteu test, Bellier test, peroxide value, sterol and triglyceride composition, steroidal hydrocarbons, wax and fatty acid alkyl esters content.

15 samples of olive oils from different producers from Croatia and Greece were collected, both from small-scale local producers and big olive oil companies. First the

polyphenol content of obtained samples was tested using colorimetric assay based on reaction of Folin Ciocalteu reagent. Various sample preparation techniques were tested, such as: dispersive solid-phase extraction and liquid-liquid extraction. The calibration curve was obtained using standard solution of gallic acid within the range 0.4-10.0 μg/mL with the correlation coefficient 0.99. The total phenolic content found in investigated samples was in the range 0.04-123.48 mg polyphenols / g olive oil.

The content of some volatile constituents in the oil, such as short-chained alcohols as methanol and ethanol, and also some esters as ethyl acetate is considered to be closely related to the Olive oil quality. The short-chained alcohols are known as products of maturation and/or fermentation of the olive drupes, which are badly handled before olive oil processing. The other cause for the presence of these volatile constituents is thought to be the prolonged shelf life of the product during which degradation processes may occur.

Therefore, a new static headspace capillary gas chromatographic method with flame ionization detection (sHSS-GC-FID) was developed for the determination of methanol, ethanol, acetone, ispopropanol and ethyl acetate as suspected volatile impurities present in extra virgin olive oils and virgin olive oils. Various analyte extraction and quantitation procedures were tested (matrix adjustment method, multiextraction method and standard addition method). The use of static headspace sampling minimized the interference of other volatile matrix components and provided satisfactory results in purity assessment of complex matrix sample of the olive oil. The developed and validated method was found adequate and appropriate for the determination of small amounts of the analytes of interest.

According to obtained results small levels of methanol (3.33-12.76 ppb), ethanol (0.04-28.91 ppb) and ethyl acetate (2.23-13.70 ppb) were found in all samples, while in some of the samples acetone and *iso*propanol were found below limit of quantitation.

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LIQUID HANDLING AND CHROMATOGRAPHY: 1-D, 2-D AND 3-D

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Omics techniques rely on chromatography. Affinity purification, protein, peptide, phosphopeptide or N-Glycan sample preparation requires time and material resources. In average, HPLC/UPLC reversed phase chromatography run could be described with 60/60 rule, where the gradient starts at near 100 % aqueous and ramps to 60 % organic solvent in 60 minutes. Multidimensional chromatography at least doubles the run time and effort by injecting the eluent from the first column onto a second column. Automated liquid handling, an off-line chromatography separation solution could be used to speed up the procedure in first chromatographic dimension, making the second chromatographic dimension faster, modular and more functional (*e.g.* setup of the instrument for 2-D chromatography requires precisely defined instrument architecture and parameters that cannot be easily changed). Liquid handling chromatography platform could change any one-dimensional instrument into multidimensional instrument or it could perform multidimensional chromatography by itself, where analysis parameters, such as accuracy, precision and repeatability remain constant.

LC/MS/MS - TECHNIQUE THAT CHANGED EVERYTHING

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LC/MS/MS technique, which combines liquid chromatography and mass spectrometry, represents market demand in order to detect, to identify and to quantify ultra-low concentrations of different pollutants in environment, toxical compound in food, metabolites in biological samples and illegal psychotropic drugs.

Within LC/MS/MS system, HPLC is directly connected to the triple mass analyzer. HPLC system is powerful tool to separate different analytes on stationary phase using liquid mobile phase, and in combination with triple mass spectrometer, the criteria for identification of unknown sample is not only retention time, but also *m*/*z* value of product and precursor ions and their ion ratios.

HPLC system is consisted of degasser unit for degassing liquid mobile phases, pumps for adjusting flow and concentration of the mobile phase mixture, autosampler for injecting the sample, column oven with the column for the separation of the analytes depending on the chemical properties of the compounds and interactions between the compounds and the stacionary phase, and of controller for communication with the PC.

Triple quadrupole (MS/MS) is consisted of ionization source, mass-analyzer and detector. In ionization source, ions are generated at atmospheric pressure. Depending on the mechanism of ion generation, three techniques are known: electrospray ionization, atmospheric pressure chemical ionization and atmospheric pressure photo ionization. Neutral molecules, after passing the ionization source, are charged and the ions are transferred from atmospheric pressure into the vacuum for mass analysis through the desolvation line. Afterwards they are focused and aligned to the quadrupole mass analyzer with the help of an electromagnetic lens system. Ions are separated by the mass/charge (*m*/*z*) ratio, and detected by electron multipliers.

The design of mass spectrometer allows detection and quantification of analytes in parts per billion and parts per trillion (ppb and ppt) concentration range. That is the reason why LC/MS/MS technique represents new vision of sensitivity, and powerful tool for receiving information of pollutants in order to protect our health, our community and our environment.

INTRODUCTION OF TUTORIAL REVIEWS ON VALIDATION OF LC/MS METHODS

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Method validation is crucial for reliable results. However, the validation of liquid chromatography-mass spectrometry (LC/MS) methods is time consuming and further complicated by many different suggestions from specific guidelines. Also many guidelines are not very specific of the LC/MS method, giving general suggestions only. This increases frustration in practical user of the method, who do not orient well in validation.

Two tutorial reviews on validation of LC/MS have been published by Kruve *et al.*: "*Tutorial review on validation of liquid chromatography–mass spectrometry methods: Part I and Part II*" (doi: 10.1016/j.aca.2015.02.017 and doi: 10.1016/j.aca.2015.02.016). The focus is on LC/MS method validation, giving an overview of the state of the art of current LC/MS method validations, drawing attention to aspects specifically connected to LC/MS method and makes suggestions for solving most problematic issues of LC/MS method validation.

CHARACTERIZATION OF NEPAFENAC DEGRADATION IMPURITIES BY TWO DIMENSIONAL UPLC COUPLED WITH LC-QTOF

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Impurities in pharmaceutical product are the unwanted chemicals that remain with the active pharmaceutical ingredient (API), or develop during production or by degradation of API. The presence of these unwanted chemicals even at small concentrations may influence the efficacy and safety of the pharmaceutical products. Different scientific researches showed that degradation products can be potentially genotoxic, mutagenic or carcinogenic, thus needing constant monitoring and impurity profiling.

Techniques such as LC/MS/MS are widely used for impurity identification in pharmaceutical industries due to their high sensitivity, selectivity, and speed of analysis. Coupling high pressure liquid chromatography (HPLC) system with quadrupole time-of-flight (Q-TOF) mass spectrometry enables accurate mass measurement of both precursor and fragment ions; however this technique is limited to the usage of volatile buffers. Additional time is required to develop an equivalent HPLC method with MS compatible mobile phase. Furthermore, it may add additional challenges and uncertainty if the impurity elution order is changed with the newly developed MS-compatible LC method. This limitation can be effectively resolved with a heart-cut approach employing two reversed phase LC conditions. Heart-cutting 2D-LC transfers fractions of interest from first to second dimension ensuring the best separation at the critical parts of the chromatogram.

In order to investigate the stability of nonsteroidal anti-inflammatory drug (NSAID) Nepafenac, the drug was subjected to forced degradation under different stress conditions such as acid and base hydrolysis, oxidation, humidity, heat and light. The aim of this stability study was the structure elucidation of unknown peaks using two dimensional chromatography coupled with quadrupole time-of-flight (Q-TOF) mass detector. Major degradation impurities were determined and the peak purity was determined by usage of selected MS detector.

INTRODUCTION TO SIZE EXCLUSION CHROMATOGRAPHY COUPLED WITH MULTI-ANGLE LIGHT SCATTERING (SEC-MALS)

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Size exclusion chromatography (SEC, also called gel permeation chromatography – GPC or gel filtration chromatography – GFC) is a column chromatography that separates molecules according to their hydrodynamic volume. It is usually used to separate large molecules or macromolecular complexes such as proteins and to determine relative molecular weights and molecular weight distributions of polymers. Separation is achieved by the differential exclusion from the pores of the packing material, of the sample molecules as they pass through a bed of porous particles. In conventional mode it is necessary to find relation between molar mass and elution volume (calibration of columns).

Static light scattering techniques measure the absolute molecular weights and the size of macromolecules. Coupling SEC with multi-angle light scattering detector (MALS) the sample is first fractionated by SEC and the separate molecules elute to a MALS detector connected on-line where the molar mass is measured directly without column calibration from the intensity of light scattered by eluting molecules. Multi-angle detection allows determining molecular weights distribution, size (radius), chain branching, aggregation, conformation of macromolecules. This information is difficult to acquire using conventional offline methods, thus making MALS one of the most powerful detection techniques in SEC.

QbD DEVELOPMENT OF UPLC METHOD FOR DETERMINATION OF DEGRADATION PRODUCTS OF BIVALIRUDINE

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An UPLC method for determination of bivalirudin in the presence of its degradation products has been developed using quality-by-design approach. Novel method development approach was applied - two software packages, Fusion AE and DryLab, were used in conjunction to obtain method that can provide best selectivity in a very short analysis time. Method development was carried out in two phases: development phase - performed using Fusin AE software and the optimization phase - conducted using DryLab software. Main goal of the development phase was to define chromatographic parameters that will separate all degradation impurities from each other and from Bivalirudine peak. For that purpose different column chemistries, type of organic mobile phase, pH of the water mobile phase, composition of the mobile phase, column temperature, flow, injection volume and the time of the gradient were evaluated. Optimization phase with DryLab software was based on the data from 12 chromatographic runs. Chromatographic parameters were adjusted to obtain same or better separation power as obtained by Fusion, but at about five times shorter run time (efficacy increased for 500%). Besides efficacy, method robustness was also improved using DryLab predictions. The developed UPLC method was validated as well, and was proven to be an effective method for separating bivalirudin from its degradation products. Forced degradation studies were performed using acid, H_2O_2, heat and light exposure as recommended by the International Conference on Harmonization (ICH).

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