UNIVERSITY OF ZAGREB

FACULTY OF CHEMICAL ENGINEERING AND TECHNOLOGY

13th International Chromatography School

BOOK OF ABSTRACTS

18th– 19th June 2012

ZAGREB, CROATIA

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PREFACE

Welcome to 13th ICS 2012

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

Ph.D. Tomislav Bolanča, assoc. prof.

We would like to thank sincerely all the lecturers and sponsors for their contributions at 13th ICS.

INVITED LECTURES

ELECTROLYTIC GENERATION OF ELUENTS FOR CONVENTIONAL–SCALE AND CAPILLARY–SCALE ION CHROMATOGRAPHY. A REVIEW

N. Avdalović, Y. Liu

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Dilute solutions of acids, bases, and salts are commonly used as chromatographic eluents in ion chromatography (IC). Traditionally, these eluents are prepared off-line by dissolving reagent-grade chemicals. The off-line preparation of eluents can be tedious and prone to operator errors, and often introduces contaminants. For example, dilute NaOH solutions are widely used as eluents in the separation of anions and can be easily contaminated by carbonate. The preparation of carbonate-free NaOH eluents is difficult because carbonate can be introduced as an impurity from the reagents or by adsorption of carbon dioxide from air. The presence of carbonate in NaOH eluents often causes an undesirable chromatographic baseline drift during the hydroxide gradient and even irreproducible retention times of target analytes and thus compromises the performance of an IC method.

Considerable efforts have been made to develop on-line electrolytic devices that utilize the electrolysis of water and charge-selective electromigration of ions through ion-exchange membranes to generate high-purity and contaminant-free acid, base, and salts eluents for ion chromatography. The use of these electrolytic eluent generators in modern IC systems has fundamentally changed how ion chromatography is practiced in many laboratories. In this presentation, we will review the development of various electrolytic eluent generators for generating high purity acid, base, and salt solutions using deionized water as the carrier stream. We will describe the principles and operation of the electrolytic eluent generators and demonstrate the advantages of using these devices in conventional-scale and capillary-scale IC separations of target analytes in various sample matrices.

CAPILLARY ION CHROMATOGRAPHY –A NEW PLATFORM FOR HIGH RESOLUTION OR HIGH THROUGHPUT SEPARATIONS OF IONIC COMPOUNDS

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HPLC applications based on capillary columns have seen an impressive progress in the last decade. As an analytical tool capillary HPLC offers the following advantages:

- Easier hyphenation with mass spectrometers
- Significant reduction of eluent consumption
- Higher mass sensitivity in comparison to conventional HPLC
- Ideal for applications with limited sample volume

Introduced in 2010, capillary ion chromatography with electrolytic eluent generation offers similar advantages, although the advantages listed above have a different significance for ion chromatography applications. The most significant advantage of ion chromatography in the capillary format is the marked reduction of eluent consumption, which allows continuous operation over a long period of time. In conventional water analysis sample volume is usually not the issue, but users investigating corrosion phenomena or metabolic pathways are benefitting from much smaller sample volumes (<< 1 µL) used in capillary IC. The higher mass sensitivity in capillary IC as compared to conventional IC has a huge impact on trace analysis, because the same concentration sensitivity can be achieved with much smaller sample volumes. Last but not least, capillary IC operating with very small flow rates improves the compatibility with modern tandem mass spectrometers.

The expanded pressure tolerance of electrolytic eluent generation in capillary IC systems up to 34.5 MPa (5000 psi) allows the use of smaller particle size ion exchangers. In combination with higher linear velocities of the mobile phase, shorter analysis times can be achieved. On the other hand, it also facilitates high resolution separations of complex samples through the use of longer separator columns. In this presentation, examples of fast and high resolution separations of inorganic and organic ions from different application areas will be shown, utilizing suppressed conductivity and MS detection as well as resin-based and monolithic stationary phases.

AUTOMATED SAMPLE PREPARATION FOR ION CHROMATOGRAPHY

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The real challenge in ion chromatography often is cleaning up samples prior to injection. Depending on the matrix, this may require a lot of time and manual work. Metrohm's Inline Sample Preparation (MISP) and advanced liquid handling put an end to this. MISP stands for techniques that allow users to fully automate the preparation of even the most difficult samples – in any matrix.

With the unique Metrohm Inline Sample Preparation («MISP») techniques, some of which have been patented, it is now possible to automate these processes in full and make each individual step traceable. The high precision and accuracy of liquid handling are based on the outstanding properties of the patented 800 Dosino.

Methods of Metrohm Inline Sample Preparation are: Ultrafiltration, Dialysis, Dilution, Extraction, Matrix elimination, Neutralization, Cation Removal, Partial Loop Injection, Preconcentration and Calibration.

The advantages are obvious: you save a significant amount of time and cost, while also increasing the reliability of your analyses.

DETERMINATION OF CHLORIDE AND SULFATE IN BIOETHANOL USING IC

T. Tomić[1], M. Milčić[2], N. Uzorinac Nasipak[1]

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[2] *Faculty of Chemical Engineering and Technology, Marulićev trg 19, 10000 Zagreb, Croatia*

Bioethanol is ethanol produced from biomass and / or biodegradable (cellulose) of waste to be used as biofuel. Bioethanol is used as a blending component in commercial gasoline fuel. Important advantage compare to fossil fuels is that bioethanol reduces greenhouse gas emissions. Bioethanol produced from agricultural products is a renewable source of energy, which unlike fossil fuels, whose reserves are finite will never deplete. This is particularly evident in relation sharply rising oil prices. Bioethanol used as a blending component in commercial gasoline fuel can be contaminated with chloride and sulfate that cause corrosion and form deposits in automobile engines. Requirements for chloride and sulfate content in ethanol were defined in EN 15376 and by that maximum chloride content should not exceed 6.0 mg/kg and maximum sulfate content should not exceed 4.0 mg/kg. If chloride and sulfate content concentrations exceed the defined limits, ethanol may be rejected as unacceptable for use as engine fuel.

Ion chromatography is a powerful tool for ion analysis in different matrix. Water is most frequently used as matrix but ethanol samples can be successfully analyzed as well. Replacement of the classic analytical method leads to the use of smaller sample content, faster analysis and multiple information in a single analysis.

In this paper ion chromatography method optimization and validation is described. All measurements were performed on Metrohm 761 compact ion chromatographic system (Metrohm, Switzerland) comprising 766 IC sample processor, eluent tank, high pressure IC pump, peristaltic pump, Metrosep A Supp 5 analytical column, chemical suppressor and conductivity detector. Flow rates were from 0.6 to 0.7 ml/min and temperature of column was set at 35 ºC. For data processing IC Net software from Metrohm was used. Before analysis all samples were filtrated through 0.45 μm regenerated cellulose membrane filter.

In method optimization different mobile phase compositions and rates were investigate as well as different column lengths. Optimal were found 0.6 ml/min flow rate, 35 minutes run time, 250 mm column length and Na2CO3/NaHCO3 mobile phase without organic modificator.

After method optimization next step was calibration. For calibration, stock standard solution was diluted to appropriate concentrations and calibration graph was constructed with eight calibration points in concentration range from 0.25 to 20 mg/l.

Validation of the method has been performed in order to confirm its validity and applicability. Selection of validation parameters depends upon the method purpose and it is carried out at the analyst's discretion, as well as according to which criteria the parameters are relevant in a particular method, all according to the analyst's own laboratory experience and in accordance with the good laboratory practice. Tested validation parameters were selectivity, linearity, repeatability, accuracy, detection limit, quantification limit and robustness. Validation has confirmed the applicability of the method for a particular purpose.

DETERMINATION OF TAURINE IN ENERGY DRINK BY IC/PAD AND LC/MS

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Taurine, also known as 2-aminoethanesulfonic acid, is the main component of many energy drinks as a tonic medicine. Furthermore, it is different from most other amino acids in that it is not incorporated into proteins. Taurine is unusual among biological molecules in being a sulfonic acid, while the vast majority of biologically occurring acids contain the more weakly acidic carboxyl group. While taurine is sometimes called an amino acid, and indeed is an acid containing an amino group, it is not an amino acid in the usual biochemical meaning of the term, which refers to compounds containing both an amino and a carboxyl group. Nevertheless, it does play many important roles in the body.

A rapid and simple method for taurine determination in energy drink was developed by anion-exchange chromatography with integrated pulsed amperometric detection and high-performance liquid chromatography coupled with single-quad mass detector (Surveyor MSQ plus, Dionex). Detection of taurine was done by ESI-MS in positive ion mode.

Sample preparation did not include derivatization step, only dilution of the sample with ultra pure water for IC determination and with 20mM ammonium formate with adjusted pH=3.2 for LC determination. Mobile phase was 5mM ammonium formate in ultra pure water and 5mM ammonium formate in methanol for LC determination and ultra pure water, 250mM NaOH and 1M NaOAc for IC determination flowing under gradient elution.

For ion chromatographic determination taurine was analysed with AminoPac PA10 column (2x250mm) and guard column (2x50mm). For LC determination taurine was analysed with Zorbax Eclipse XDB C18 column (2.1x150mm, 3.5µm).

The recoveries were in the range of 90.8 – 108.4% and 87.6-95.5%, the precision as standard deviation was 6.27% and 8.41%, the linearity as a coefficient of correlation value was 0.9991 and 0.9909 for IC and LC respectively. The content of taurine measured in commercial energy drinks was 400-4000mg/l.

EU and Serbian legislation have defined a maximum concentration of taurine in energy drinks which is 4000mg/l.

References:

1. Cataldi TR, Telesca G., Bianco G. Anal Bioanal Chem (2004) 378:804-810, Improved determination of taurine by high-performance anion-exchange chromatography with integrated pulsed amperometric detection (HPAEC-IPAD).

11TH PROFICIENCY TEST: CRITICAL REVIEW

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Proficiency testing is the use of inter-laboratory comparisons to determine the performance of individual laboratory for specific tests or measurements and to monitor a laboratory's performance. Participation in proficiency testing schemes provides laboratories with an objective means of assessing and demonstrating the reliability of the data they are producing. The proficiency testing as a part of International Chromatography School has 11 years tradition.

For current test 7 laboratories submitted the results of cation and anion analysis for given sample. The data were analyzed and the final report was sent back to the participants. In this critical review the anonymous (coded) list of all valid data and the statistical results are presented.

COMPREHENSIVE TWO DIMENSIONAL GAS CHROMATOGRAPHY COUPLED TO TIME-OF-FLIGHT MASS SPECTROMETRY IN CHALLENGING APPLICATIONS

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Comprehensive two-dimensional gas chromatography (GCxGC) has recently found its important role in many gas chromatographic applications thanks to its enormous separation power. Unlike "heart-cutting" techniques, in GCxGC the whole chromatograph is subjected to two independent separation mechanisms. In **Figure 1**. the schematics of a GCxGC device is shown. In GCxGC, two columns of different selectivity are connected via a thermal modulator, which cuts small portions of the first column eluate, focuses them to sharp zones and sends them onto the second column, where a very fast "flash" separation is performed – see **Figure 2**.

Besides enhanced separation power, this technique provides the advantage of structured two-dimensional chromatograms, in which each compound of a sample occupies a position according to its retention behavior on the two columns with different selectivity (e.g. volatility x polarity).

In the applications, where the confirmation of analyte identity is necessary, mass spectrometry is a desirable detection technique. In GCxGC, extremely narrow peaks (0.1 – 0.2 s) are produced on the second dimension column. Therefore the detection technique used has to be fast enough to describe these narrow peaks properly. In the family of mass spectrometers only time-of-flight MS reaches the acquisition rates needed, since its maximum acquisition rate is 500 Hz, which is cca 10 x higher than for other MS detectors.

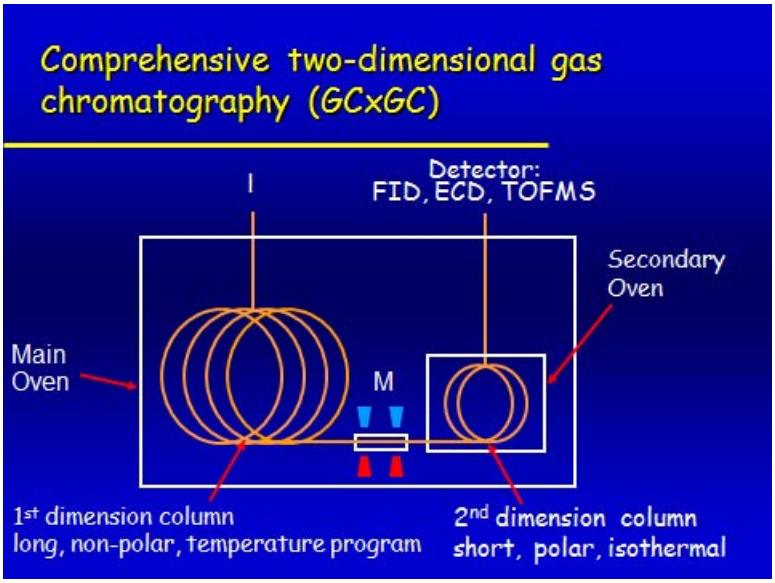

Figure 1. The Schematics of a GCxGC instrument

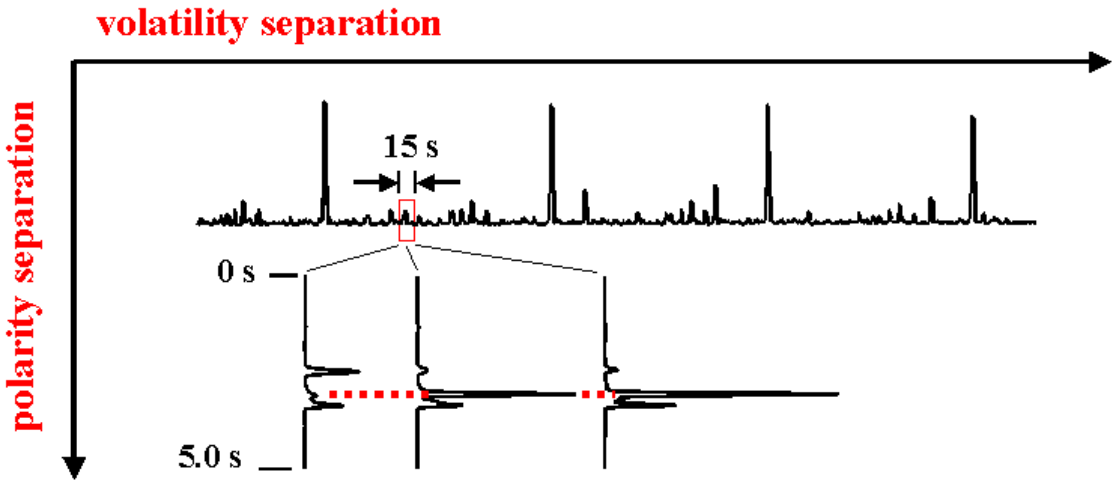

Figure 2. Principle of GCxGC operation

GCxGC-TOF MS has been shown to be a powerful instrumental technique in both scientific and routine application in the areas of petrochemical analysis, pesticide residues analysis, metabolomics and others.

Examples of the above application will be shown in the presentation.

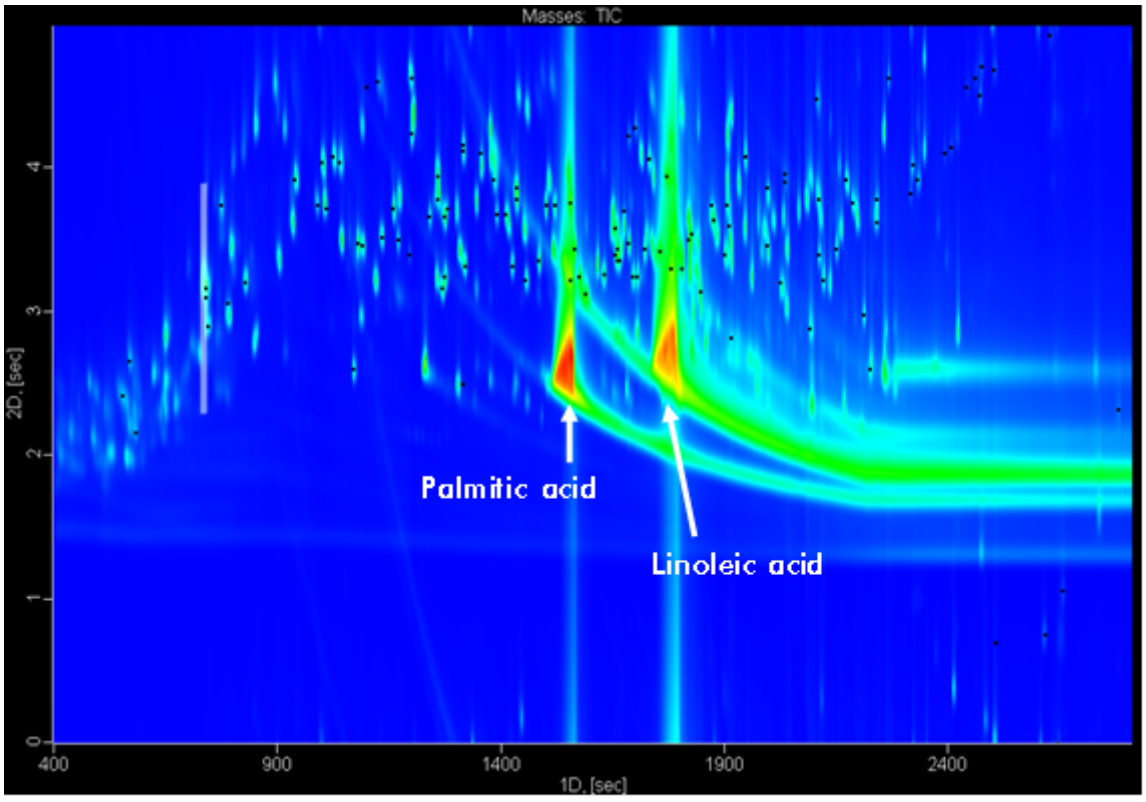

Figure 3. GCxGC contour plot from the analysis of 215 pesticides in grains. Black dots correspond to spiked pesticides. Thanks to second (polar) chromatography dimension the pesticides are separated from matrix background.

APPLICATION OF GC x GC TECHNIQUES FOR DIESEL FUEL

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Gas chromatography is used for separation, qualitative and quantitative analysis of complex mixtures. It is achieved better resolution in less time than most analytical techniques used today. However, for very complex samples, with large number of components, the major constraint is overlapping of peaks. Their separation can be very difficult, sometimes impossible, and many components can remain fully masked and thus unidentified.

Diesel fuel is a complex of hydrocarbons mixture derived from various refinery products and additives. The properties of diesel fuel depend on the composition of saturated and aromatic hydrocarbons. The quality of diesel fuel depends on its hydrocarbon composition, which is of great importance in planning and optimization of refining processes as well as caring for the environment. The latter is mainly related to aromatics, especially polycyclic hydrocarbons. Knowing the composition of aromatic hydrocarbons it is possible to predict the quality and environment-friendly diesel fuel. One of the important ecological characteristics of diesel fuel is the amount of polycyclic aromatic hydrocarbons (PAHs). The quality of fuel in the Republic of Croatia is regulated by the Regulation on the quality of liquid petroleum fuels. Quality requirements are specified INA standards which are harmonized with EU legislation.

Comprehensive two-dimensional gas chromatography (GCxGC) is one of the most powerful analytical techniques for the analysis of organic compounds in complex mixture.
It is faster, more accurate and more precise than 1D chromatography in separation and the analysis of complex samples such as oil, flavorings. Comprehensive gas chromatography is the result of a combination of two nearly independent systems.

To understand the working principle of such a complex system it is necessary to know the terminology as it is comprehensive and heart cuting coupling, modulation, ortogonality, peak capacity. Technique is based on continuous collection of the effluent from the first column and periodic reinjection of small parts of the effluent to a second dimension column of different polarity in very fast and very narrow zones. In this way the separation achieved in the first column is preserved and additional separation in the second column is accomplished. The revolutionary aspect of GCxGC is that the entire sample is subjected to two distinct analytical separations. This principle of operation and the information that it provides is of great importance in the petroleum industry, including the analysis of diesel fuel. Diesel fuel samples can contain thousands of compounds and in most of the situations all of them are analytes of interest. If the separation was successful, a chromatogram is obtained in which each spot represents one component of the sample. The chromatogram obtained by well-defined shapes can be separated in groups of compounds of interest (aromatics, aliphatics ...). Software provides graphical separation of compounds. After saving and naming such graphic separation a classified template is obtained. Once created, classified template can be used as a comparison of the chromatogram of the same type of sample (diesel fuel, oil...). The result can also be presented in 3D display.

In addition to the qualitative separation GCxGC also provides quantitative results of group determination of diesel fuel. Groups of compounds that can be determined by analyzing

diesel fuel are aliphatic and aromatic hydrocarbons. Among the aliphatics n-alkanes, iso-alkanes and cyclo-alkanes can be recognized. Aromatic hydrocarbons include mono-, di-aromatics, and three (+) aromatics. Polycyclic aromatic hydrocarbons represent the sum of di-, and three (+) aromatics. Fuels with a high aliphatic hydrocarbons have better combustion performance while with a high content of aromatic hydrocarbons have poor combustion properties and a greater tendency to form particles of soot and smoke.

The main features of GCxGC technique is the possibility to provide detailed information of complex mixtures. Peaks are classified to represent groups of compounds, which gives an excellent overview of group composition of the sample. It offers excellent resolution, high peak capacity and increased sensitivity compared to the 1D GC, which gives more accurate and precise results. It gives a clear picture of a very complex mixture based on differences in boiling point and polarity of the components of the mixture. As for the diesel samples, GCxGC technique is suitable for qualitative and quantitative group determination of diesel fuel. Despite the complexity of chromatographic technique, it gives much more information than any other analytical technique because of good resolution and sensitivity. The advantages are evident in the fact that no prior sample preparation is required and very small amount of sample. Clearly, GCxGC offers great potential for the further development in application for complex hydrocarbons samples.

CHARACTERIZATION OF ESSENTIAL OILS BY GAS CHROMATOGRAPHY - MASS SPECTROMETRY

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Essential oil analysis comprises a wide range of techniques that are used for extraction, concentration, chromatography, and characterization of constituents, and most of them are presented in this work.

Essential oils are valuable natural products that are popular nowadays in the world due to their effects on the health conditions of human beings and their role in preventing and curing diseases. The chemical quality of the essential oils is determined by their composition as well as by the complex aroma features. The methods of essential oil analysis are employed by natural product chemists, pharmacologists and biologists to conduct their scientific research and to valorize natural products. Standardization of some of these methods is therefore desirable to permit more comprehensive evaluation of plant oils, and greater comparability of the results obtained by different investigators.

Steam distillation, hydrodistillation, and headspace sampling are mostly used for sample preparation and concentration. The major chromatographic technique is gas chromatography, and tandem techniques of chromatography linked to further chromatography and spectroscopy, allow much increased resolution, and greater ease of characterization of essential oils. Chromatography operating conditions and stationary phases, and techniques for solute identification are laid out.

Among different techniques for analysis of essential oils, gas chromatography-mass spectrometry (GC-MS) is the most important one in recent years. Mass-spectral tools can be very useful in identifying essential oil ingredients. Moreover, the use of fast-GC, fast-GC-qMS analysis, enantioselective GC, multidimensional GC techniques and GC–isotopic ratio mass spectrometer (GC-IRMS) for analysis and quantization has been intensively employed in latest years.

The chromatographic system can provide retention data which serve as complementary information for the positive identification of resolved components. The values of retention data are mainly calculated by applying the equations proposed by Kováts, for isothermal analysis, and van den Dool and Kratz, for programmed gas chromatographic runs. The use of retention indices in the flavor and fragrance field is well-documented, and they are widely applied for the comparison of results between laboratories, as well as to characterize stationary phases.

However, there are some fundamental problems in GC-MS analysis including baseline drift, spectral background, noise, low S/N (signal to noise) ratio, changes in the peak shapes and co-elution. Latest multivariate curve resolution (MCR) approaches cope with ongoing challenges and are able to handle these problems.

APPLICATION OF GAS CHROMATOGRAPHY IN PETROLEUM INDUSTRY

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Chromatography in all forms is being widely used throughout the industry, and in comparison with other analytical techniques, application of gas chromatography in petroleum industry indeed shows how powerful this technique could be. The main advantage of this technique is its ability to analyze very complex samples.

It could be said that gas chromatography started its development in petroleum industry knowing that on the first international symposium on vapor phase (gas) chromatography ever held (1956) nearly all of the application papers were of some significance to the petroleum industry. First applications described analysis of refinery gas streams, distribution of aromatic compounds in naphtas, solvent analysis and analysis of samples taken from internal combustion engines. Growth of gas chromatography in petroleum industry enhanced with availability of commercial instrumentation during 50's.By the end of that decade analysis of C5 to C6 saturated hydrocarbons was extended up to C7 and with an introduction of highly efficient packed columns separation of C7 and C8 paraffin isomers was performed.

At the beginning of the next decade, a new technique of open tubular (capillary) gas chromatography had been introduced which made chromatography analyses even more efficient. A milestone was achieved when a method for determination of C3 through C12 hydrocarbons in a full range gasoline was published.[(1)]

Nowadays, gas chromatography in petroleum industry presents a powerful tool in achieving both qualitative and quantitative information about various samples composition, product quality and pollutants monitoring.

Various types of instruments and analyzers represent great utensils for sample compound determination on a detailed and simple way.

By different gas chromatographic test methods and sample preparation techniques, samples from gasoline to water can be analyzed.

Gasolines are being analyzed through Detailed hydrocarbon analysis (DHA) which gives information about individual and group composition for different types of gasolines while organically bounded oxygen compounds (oxgenates) and total oxygen content are determined with two column system analyzer.

By means of simulated distillation hydrocarbon boiling range distribution of middle and heavy oil fractions can be given on a simple and quick way.

Biodiesel samples are tested for meeting the requirements prescribed by European standard for biodizel quality, where fatty acid methy esther, methanol, mono-,di-, triglycerides and free glycerol content is determined.

Certain GC analyses are also related to waste and surface water pollutants monitoring such as determination of hydrocarbon oil index and determination of benzene and some derivates.

Wide range of applications show how gas chromatography, either basic or in coupled systems (GCMS, GCxGC), can follow the challenges in petroleum industry.

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1. D.L. Camin, A.J. Raymond, *Chromatography in petroleum industry*, Journal of chromatographic science, **11**(1973)625-636.

UPC² - EXPENDING THE BOUNDARIES OF LC & GC SEPARATIONS

H. Boiteux

Waters European Headquarters, 5 rue Jacques Monod, 78280 Guyancourt, France

A new category of separation science, UltraPerformance Convergence Chromatography (UPC²™), offers an additional solution to the challenging separations encountered in laboratories. Compressed CO_2, the primary mobile phase for UPC², offers major advantages over the liquid mobile phase or carrier gases that are used with LC and GC. For one, CO_2 alone, or in combination with a co-solvent, is a low viscosity mobile phase that achieves higher diffusion rates and enhanced mass transfer than liquids used in HPLC. For another, when compared to GC, CO_2 alone is a mobile phase that allows separations to occur at a much lower temperature.

Along with sub-2 μm particle column chemistries, Waters ACQUITY UPC² System gives scientists the ability to precisely vary mobile phase strength, pressure, and temperature. It is routine to acquire the same high quality data as in UPLC applications, but the different mechanism of separation (including chiral separation) offers the ability to fine-tune the resolving power as well as a completely different choice of selectivities. The qualitative and quantitative capability of the system are unparalleled compared with previous SFC systems and this new technology provides a powerful complimentary technique for the separation of complex mixtures.

Whether analyzing natural products, traditional medicines, drugs, food additives or contaminants, pesticides, surfactants, polymer additives, or biofuels, the ACQUITY UPC² System now takes its place alongside LC and GC as a powerful complementary technique, applicable to a large number of compounds with a wide range of polarities.

ADVANCED CONFIGURATIONS IN HPLC

J. Weiss

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Maximizing sample throughput while at the same time saving additional resources and laboratory space is a key issue in HPLC. This created demand for highly productive LC and LC-MS solutions without sacrificing robustness. A common approach to increase sample throughput is summarized under the umbrella term of U-HPLC. It requires changing key method parameters such as column dimensions, particle sizes, flow rates, and column temperature. In a regulated environment, this implies revalidation of methods and revision of corresponding documentation. Although software tools for method transfer are available today, a number of HPLC users do not implement speed optimized methods because of the workload coming along with it.

On the other hand, strategies exist to increase sample throughput while leaving key method variables untouched. Dual-column operation, with off-line column reconditioning (tandem operation) is such a strategy. It is applicable to gradient methods and typically increases throughput by 35 to 50%. Parallel chromatography with a dual-gradient HPLC system is another suitable approach. The parallel chromatography solution nearly doubles sample throughput for either isocratic or gradient methods. A dual-gradient HPLC system can also be used for an automated application switching. In this approach, the operator sets up both methods. After the system automatically starts and equilibrates, the first method runs and then the system automatically switches to the second method without any additional operator intervention. This approach frees operator time und thus boosts laboratory productivity.

Solid-phase extraction (SPE), commonly used off-line to isolate the analytes of interest from complex matrices, can also be completely automated with a dual-gradient HPLC system. However, the barrier to work with on-line SPE-LC is high, because the creation of an instrument method that can automate all steps is complicated. With the help of an on-line SPE-LC wizard provided by the Chromeleon CDS these problems are eliminated as the operator is guided through all steps required for creating and optimizing such method.

Last but not least, an advanced configuration exists that can be used to completely automate HPLC method development, which is still considered as one of the crucial bottlenecks that impedes laboratory productivity. Due to the vast number of separator columns, mobile phase additives and pH values applicable in reversed-phase chromatography, manual method development is very time-consuming. With the progress in U-HPLC, ultrafast method development techniques became available that allow a more comprehensive screening along any possible combination of column, eluent, and temperature.

This presentation discusses instrument and software requirements for LC solutions based on dual configurations that greatly facilitate the use of these techniques.

FAST METHOD DEVELOPMENT USING QbD APPROACH

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Throughout the drug development process, methods are developed at various stages, often consisting of samples that vary in complexity. Due to the inherent nature of this process, costly and time-consuming redundant efforts may take place across an organization.

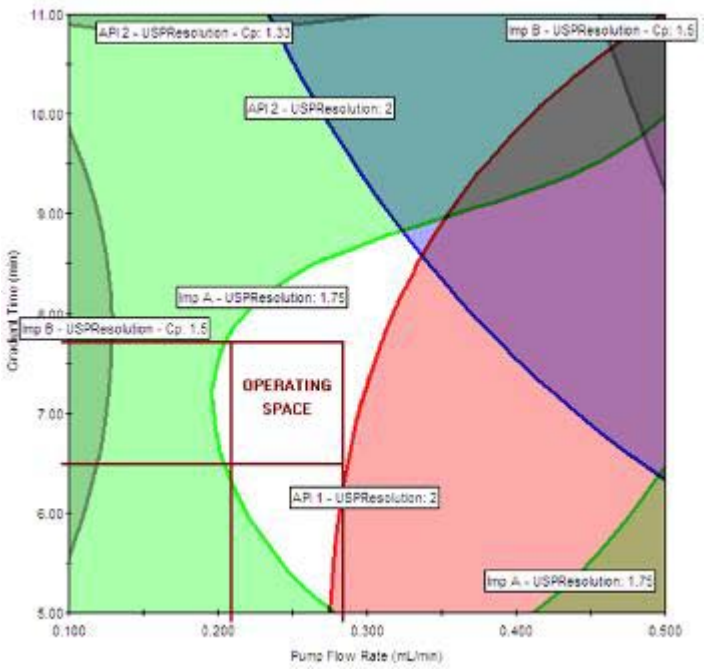
Many different approaches are applied to develop chromatographic methods today, including trial and error, method and column scouting, and software-based approaches such as first-principles approach and simplex optimization procedures. These approaches cannot, however, determine the effects of complex interactions between method variables or measure method robustness. Approaching method development from a Quality by Design (QbD) can address these challenges.

Implement an efficient two-phase method development strategy

To quickly develop optimized LC methods, Fusion Method development uses a two-phase strategy:

- **Phase 1 - Column and Solvent Screening**
  Rapid screening experiments study the major effectors of LC method performance, including column type, pH, and mobile phase composition. Fusion automates the control of internal and external column and solvent switching valves to enable walk-away experimentation.

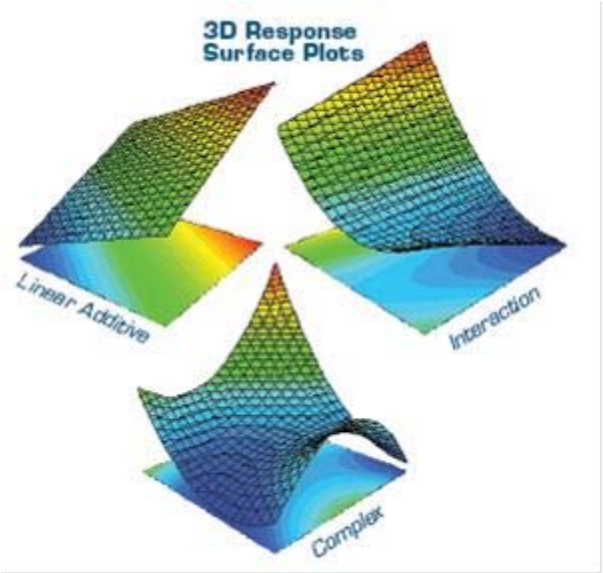
- **Phase 2 - Method Optimization**
  Phase 2 experiments use the results from Phase 1 plus additional variables with tighter ranges to determine the optimum LC method. Fusion also applies a unique combination of Monte Carlo Simulation and Process Capability statistics to quantitatively evaluate method robustness.



Analyze data without tracking peaks

Fusion Method Development's patented Trend Response™ operators automatically characterize the quality of chromatographic separations and eliminate the need for laborious and often inaccurate peak tracking during initial rapid screening experiments.

Fusion Method Development analyzes and models critical method performance characteristics easily. Response surface plots show the combined effects of variables on key chromatographic responses such as peak resolution, tailing, and retention. Color gradation represents the magnitude of interactions with curvature indicating the type of interaction.



Response surface plots show the combined effects of variables on key chromatographic responses.

Automatically predict mean method performance and robustness

Chromatographic data are used to generate parameter-effect models which are displayed numerically using the Automated Optimizer and graphically as Overlay graphs for easy interpretation.

User-defined method performance goals are quickly tested with the Automated Optimizer wizard that searches for the LC method that meets all performance goals simultaneously and displays these as method predictions.

Fusion Method Development overlay graphs predict mean method performance and robustness. These graphs show method variable ranges that define the QbD knowledge and design spaces. Non-shaded areas correspond to variable ranges that exceed all goals for mean method performance.

The Robustness Simulator™ incorporates robustness testing into LC method development. This patented simulator computes and models the impact of key chromatographic parameters on method robustness. Using the Robustness simulator identifies methods optimized for both mean performance and robustness without the need for additional experiments.

Visualize method performance and robustness with Fusion Method Development's overlay graphics. The color-coded response maps define the QbD Knowledge, Design and Operating Spaces. With these spaces defined, users can automatically predict robust LC methods.

LATEST TRENDS IN LIQUID CHROMATOGRAPHY – CORE SHELL TECHNOLOGY AND ULTRA - HIGH EFFICIENCY

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Core-shell technology is State-of-the-art Particle Technology for increasing LC productivity and achieving optimal HPLC/UHPLC performance. With this technology, chemists are able to shorten analysis times without sacrificing resolution, and get greater peak heights for improved sensitivity and better resolution. Those advanced HPLC column technologies seek to achieve very high plate counts or very low plate height. Core-shell particle is latest step in evolution of LC particle technology and has fully porous shell alongside with solid core. Columns with core-shell particles are optimized for lower plate height. In Van Deemter equation, factors A (Eddy's diffusion), B (Longitudinal diffusion) and C (Resistance to mass transfer) are minimized. Selectivity range of the Core-shell columns is similar to classical fully porous columns (C8, C18, PFP, Phenyl-Hexyl, Hilic) and dimensions of the core-shell particle inside different columns varies from 1.7 µm to 2.7 µm.

HOW TO CHOOSE APPROPRIATE STATIONARY PHASE OF LC COLUMN IN METHOD TRANSFER FROM HPLC TO UPLC

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Modern chromatographic material requirements for LC columns can be divided by physical and chemical characteristics.

Concerning physical properties of stationary phase particle size, pore size and volume are important in method transfer from HPLC to UPLC separations. Most important for method transfer to UPLC is particle size, but does not dictate the whole performance of the column.

Chemical characteristics such as stationary phase purity, surface chemistry and ligand concentration are crucial for achieving desired separation for analytes of interest. Depending on type of liquid chromatography, either HPLC or UPLC, LC analyst should consider maximum back pressure that is expected during the separation. To achieve best possible stability of stationary phase during multiple separations on the same LC column, backpressure is an important critical factor that can influence the stability of analysis. To reach the desired resolution between analytes of interest, ligand type, efficiency and selectivity are crucial for good separation, especially when sample is a mixture of analytes with different chemical properties.

When choosing the appropriate column for method transfer, analyst should consider also the manufacturer of the column and inquire how is stationary phase manufacturing process controlled for batch to batch reproducibility to ensure the final chromatographic product produces repeatable and reproducible results. Having high level of consistency enables analyst to minimize the risk of method variation due to differences caused by chromatographic media batch or column inconsistency in efforts to improve method robustness.

However, considering all above mentioned characteristics of stationary phase of LC column still does not result in successful method transfer. Liquid chromatograph used as a system of choice has individual system volume that has to be measured before choosing correct column dimensions for method transfer. Column volume is especially important in gradient separations. Combining all these factors lead to successful method transfer and lead to robust method and reproducible results.

Key words: LC columns, stationary phase, HPLC, UPLC, method transfer

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FAST AND HIGH RESOLUTION TOF MS IN LC-MS APPLICATIONS IN METABOLOMICS, PESTICIDES AND TOXICOLOGY

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Time-of-flight mass spectrometry is MS technique of growing use and potential. The principle of time-of-flight is very simple, based on the relation between mass of an ion and its velocity of flight in a vaccum flight tube.

Time-of-flight technology has gone through a lot of development in the last decade, thus nowadays these MS detectors can offer extraordinary performance as concerns their acquisition rates, mass resolution, full mass spectral information. Until recently, two types of TOF MS were available in the market: high resolution (but low acquisition rates) OR high speed (but low resolution).

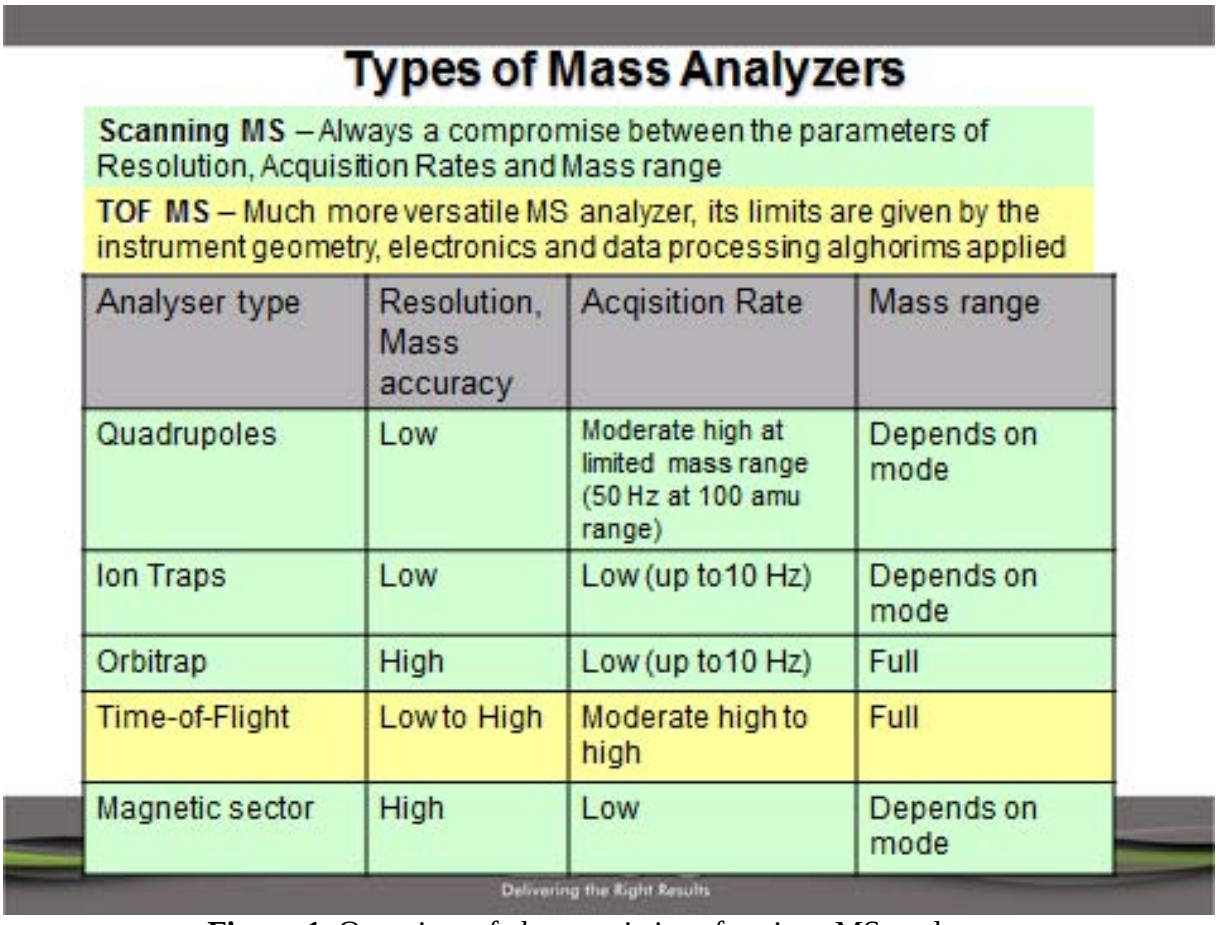

Figure 1. Overview of characteristics of various MS analyzers

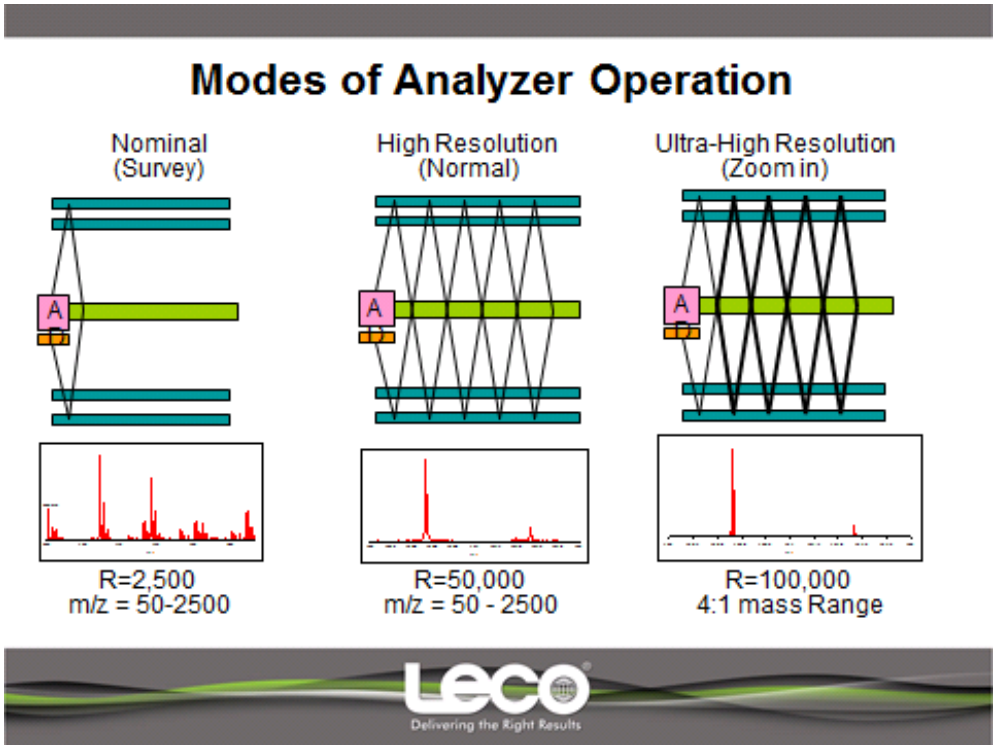

Figure 2. Schematics of Multireflecting TOF MS with Folded Flight Path technology

Last year LECO's Folded Flight Path™ (FFP™) TOF-MS technology has been introduced at Pittcon exhibition. This unique concept utilizes multiple reflections of ions in the mass analyzer, which leads to significant total length of their flight trajectory and hence high mass resolution is achieved. At the same time the detector is very fast, i.e. can collect up to 200 full spectra/second. In Figure 2 a schematics of Multireflecting TOF MS with Folded Flight Path technology is shown. This MS detector can work in three modes of operation: Nominal resolution, High Resolution and Ultra High Resolution. In High resolution mode a mass resolution of 50 000 is achieved with full mass range. In Ultra High Resolution Mode a mass resolution of 100 000 is achieved with limited mass range (1:4, low end mass:high end mass, e.g. 150-600). In all modes ultra high acquisition rates (up to 200 Hz) are achieved without compromises in resolution or mass range.

High mass resolution in GC-MS and LC-MS applications brings significant benefits over a conventional low resolution MS:

- more confident identification based on exact mass
- structural elucidation based on exact mass and fine isotopic structure
- unknowns identification
- elimination of chemical noise in the analysis of target compounds in complex matrices

In this presentation the basic principles of multireflecting TOF MS, as well as applications in the fields of metabolomics, pesticides and toxicology will be shown.

PHOTOCATALYTIC DEGRADATION OF SULFA DRUGS

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Pharmaceuticals are chemical substances which are used to treat and prevent disease in human and in animals. In farming they are also used as additives to feedstock in order to promote growth in animals. The presence of pharmaceuticals in the environment raises a big concern due to their continuous input in the environment via different pathways. They pose a toxicological risk to different non-target organisms and in that sense they can lead to development of antibiotic resistant strains of bacteria [1, 2].

Sulfonamides are widely used because of their low cost and relative efficiency against many common bacterial infections. They are highly soluble in water and due to that they are present in different aquatic systems what has been confirmed in many researches [3]. Advanced oxidation processes have been effective in the degradation of organic pollutants and accordingly they can be applied for removal of antibiotics during the waste water treatment.

In this work sulfamethazine was chosen as the representative of the group of sulfonamides. Aqueous solution of sulfamethazine was photolytically and photocatalytically degraded by UV light with different predominant wavelengths (254 and 365 nm) and by nanostructured sol – gel TiO_2 film. As a photoreactor a borosilicate glass tube with immobilized TiO_2 film was used. HPLC-ESI(+)-MS Agilent 1200 was used to monitor degradation of sulfamethazine and the appearance of degradation products. For chromatographic analysis polar embedded C18 Synergy Fusion column (4 μm, 150 x 2.0 mm, Phenomenex) was used. The analysis was performed using 0.1% formic acid in water and 0.1% formic acid in acetonitrile in gradient elution mode. Structural formulas of the degradation products were also proposed based on spectral analysis obtained on mass spectrometer.

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"ORPHAN" DRUG DEVELOPMENT AND PHARMACOKINETIC PROFILING BY CHROMATOGRAPHIC TECHNIQUES

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An orphan drug is a pharmaceutical preparation that has been developed specifically to treat a rare medical condition. A rare disease, also referred to as an "orphan disease," is any disease that affects a small percentage of the population. Most of these diseases are genetic, and therefore are present throughout the person's entire life, even if symptoms do not immediately appear. It should be pointed out that many rare diseases appear early in life, and about 30 percent of children with rare diseases will die before reaching their 50th birthday. The market for orphan drug is small and thus largely unprofitable; therefore government interventions and financial support are often required to motivate manufacturers and universities to address the need for an orphan drug discovery. Orphan drugs generally follow the same regulatory development path as any other pharmaceutical product, in which testing focuses on drug discovery, pharmacokinetics and pharmacodynamics, as well as, manufacturing and quality control of bulk drug and pharmaceutical formulation.

Mitotane is the chemotherapeutic agent used in the treatment of adrenocortical carcinoma, a quite rare but aggressive malignancy with an incompletely understood pathogenesis and a poor prognosis. In 2002 mitotane obtained EMEA approval for the orphan drug status. Therefore new findings regarding this drug are of exceptional medical relevance for adrenocortical carcinoma patients.

Lipophilicity represents the affinity of a drug for a lipophilic environment. It is a fundamental physico-chemical property related to pharmaceutical and biomedical activity of drugs. It can be determined in different ways, but the chromatographic determination of lipophilicity is currently one of the major approaches present in the literature due to sophisticated instrumentation and a wide range of measurable lipophilicity values. In this presentation determination of lipophilicity of mitotane by RP-HPLC technique will be described.

Quality control of pharmaceutical formulations is an important task in the pharmaceutical industry. It not only protects the manufacturer against compensation claims, but also guarantees patients a safe and effective product. Therefore, a novel, simple, fast and validated method for quality control of mitotane in pharmaceutical formulations by HPLC technique has been proposed.

Finally, a simple, fast and reliable method for the simultaneously determination of mitotane and its main metabolites in human plasma, red cell and urine samples using a SPE-HPLC-DAD technique will be presented. The proposed method was applied for identification and quantification of mitotane and its metabolites in biofluid samples from adrenocortical carcinoma patient. Furthermore, the method was applied to investigate the pharmacokinetic profile of mitotane after oral dose of 500 mg. Obtained pharmacokinetic profile for the patient involved in this study has shown a high plasma concentration of mitotane while none of investigated metabolites was found. As mitotane belongs to the class of drugs that require metabolic transformation for therapeutic action, the polymorphism of CYP2C9 and CYP2C19

enzymes by real-time PCR analysis was performed in order to investigate whether enzymes' polymorphism could be related to the metabolism of mitotane.

QUALITY CONTROL OF DIETARY SUPPLEMENTS BY CHROMATOGRAPHY AND CAPILLARY ELECTROPHORESIS

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Quality control monitoring ensures the safety, efficacy and quality of pharmaceutical preparations. It consists of a regular testing of the quality of medicines regarding the identification and determination of active compounds as well as drug-related impurities. Dietary supplements (also known as food supplements) are concentrated sources of nutrients or other substances with a nutritional or physiological effect whose purpose is to supplement the normal diet. Dietary supplements are finding a niche both in the fields of preventive nutrition and alternative medicine, and receive a growing recognition as a good means for health maintenance. They are marketed in dose form i.e. as pills, tablets, capsules, liquids in measured doses etc. Unlike medicines which are a strongly regulated field, with quality, safety and efficacy insurance, Good Manufacturing and Laboratory practice, dietary supplements are under Food regulatory agencies. There is no uniform legislation in the food supplements area despite their ever growing popularity and presence on the market. Health safety, nutritional value and laboratory control of declared content is very rare. There is no risk assessment and pharmacovigilance. Declaration of content is insufficient without special warnings. Since these products are more often used then medicines, on daily basis, consumed by children, pregnant women and old people, without the advice from a doctor or a pharmacist, quality control of these products is of great importance.

Today advance analytical techniques can be employed for identification and quantification of active compounds and impurities. Special emphasis should be given to separation techniques such as high performance liquid chromatography (HPLC), gas chromatography (GC) and capillary electrophoresis (CE). In this presentation new HPLC-DAD, CE-DAD, HPLC-DAD-FLD-MS^n and HSS-GC-FID methods with application to different food supplements, developed by our group, will be shown.

Artichoke is considered functional food because of its high content of minerals, vitamins and phytonutrients. Clinial studies have shown antioxidative, hepatoprotective, antiemetic, spasmolytic, carminative, diuretic, choleretic, anti cholestatic activity of artichoke. Due to its high content of polyphenol cinarin it lowers cholesterol and fat levels in blood. A simple HPLC-DAD method was developed for investigation of cinarin presents in artichoke food supplements available in community pharmacies, drug stores and organic food stores.

Red yeast rice products are an excellent dietetic product for lowering cholesterol and LDL levels in plasma and therefore it is used by millions of people around the world. Main active ingredient is monakolin K, which is actually lovastatin, registered as a medicine in 1987. However, red yeast rice products are food supplements so they do not comply to strict legislation as medicines do. Therefore significant differences among manufactures and even between lots of the same manufacture appear. New scientific research shows that a highly nefrotoxic compound, citrinin, is also present in red yeast rice products. In order to ensure efficacy and safety of red yeast rice products, standardization of lovastatin content and determination of citrinin is necessary. Two new methods for identification and quantitation of

monakolin K, monakolin K acid and citrinin in red yeast rice products were developed and validated. A simple, fast, low-cost and environmental friendly micellar electrokinetic chromatography method (MEKC) was employed. A second method used a high performance liquid chromatography technique coupled to three detectors, diode array (DAD), fluorescence (FLD) and electro spray ionization ion trap analyzer (ESI-MS^n) to ensure selectivity, sensitivity and enabling identification of unknown ingredients.

Impurities are the unwished chemicals which may have unwanted pharmacological effects; they may be toxic and can influence the stability of the product. In ethanolic food supplements methanol is present as the main volatile impurity. It is a highly toxic alcohol whose ingestion or inhalation can cause damage of central nervous system, blindness and even death. A new headspace gas chromatographic method with flame ionization detection (HSS-GC-FID) was developed and validated for the determination of methanol as the main volatile impurity present in diverse alcoholic herbal extracts and tinctures as well as propolis tincture in ethanol. Because of the miscellaneous food supplements products with complex matrices, headspace sampler (HSS) was employed minimizing the interference of other volatile matrix components and providing satisfactory results in purity assessment of different complex samples. In the headspace sampling, a liquid or a solid sample is placed in a sealed vial, which is then thermostated until a thermodynamic equilibrium between the sample and gas phase is reached. Afterwards, a known aliquot of the gas is introduced into the GC for analysis. Consequently, only volatile components extracted into the gas phase at optimized conditions are introduced into the GC system, resulting in an extended lifetime of the column, significantly reduced analysis time, enhanced selectivity and sensitivity.

IN-HOUSE VALIDATION OF A METHOD FOR THE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY DETERMINATION OF BISPHENOL A IN LANDFILL LEACHATES

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Municipal landfills may be sources of a wide range of compounds with environmental, wildlife and human health concern. Bisphenol A (BPA) is an organic pollutant, commonly used in the production of polycarbonate plastics and epoxy resin. It is toxic to aquatic organisms and estrogenic active and thus belongs to a group of so called endocrine disrupting compounds (EDCs) as the representative of xenoestrogens. One of the most likely sources of BPA found in environmental samples is the leachate from waste landfills. When waste plastics containing BPA are buried in a landfill, a hydrolytic or leaching process may occur to release BPA. It is frequently present in municipal landfill leachates where as high as 17.000 $\mu g\ L^{-1}$ can be found. The presence of different organic pollutants in environment has adverse effects on soil, water and atmosphere inhabitants. Thus, measurements of bisphenol A concentration in effluents from municipal landfills are highly relevant.

The use of advanced oxidation processes (AOPs) such as photocatalysis, ozone based technologies and ultrasound oxidation have been investigated in respect to their ability to increase the biodegradability as well as detoxification of polar and hydrophilic chemicals. To monitor the efficiency of bisphenol A elimination from landfill leachate by using various advanced oxidation processes, an analytical procedure with corresponding quality of measurements should be developed and validated in order to enable potential subsequent kinetic and mechanistic dissemination. Therefore, for consistent interpretation of measurement results it is necessary to evaluate the confidence that can be placed in. This can be done by integration of metrological principles to measurement methods applied.

Metrology is science of measurements and basic aim of metrology is to ensure the comparability of measurements. The term “comparable” is used to mean that measurements have three important features: measurements made at different laboratories, different times and different methods can be compared. Comparability of measurements is assured by establishment of measurement traceability, use of validated methods and measurement uncertainty evaluation. Measurement traceability is defined as *“property of a measurement result whereby the result can be related to a stated reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty”*. The key requirement of traceability is full understanding of the uncertainty of the entire chemical measurement procedure. Measurement uncertainty *»is parameter characterizing the dispersion of the quantity values being attributed to a measurand, based on the information used«*. The ISO definition of validation is *»confirmation by examination and provision of objective evidence that the particular requirements of a specified intended use are fulfilled«*. Method validation is needed to confirm »the fitness for purpose« of a particular analytical method, i.e. to demonstrate that a defined method protocol, applicable to a specified type of test material and to a defined concentration rate of the analyte is fit for a particular analytical purpose. This analytical purpose reflects the achievement of analytical results with an acceptable standard of accuracy. Validation is thus the tool used to demonstrate that a specific

analytical method actually measures what it is intended to measure, and thus is suitable for its intended purpose. Before any method validation is started, the scope of validation must be fixed, comprising both the "analytical system" and the "analytical requirement". A description of the analytical system includes the purpose and the type of method, the type and the concentration range of analyte being measured, the types of material or matrices for which the method is applied, and a method protocol. Reliable analytical results are based on a clear specification of the analytical requirement.

In this study, we developed an analytical protocol for the determination of bisphenol A in landfill leachates by using high performance liquid chromatography (HPLC). The aim is also to identify the major sources of uncertainty with the goal of potentially reducing the overall measurement uncertainty of the result. Considering that incomplete or misleading in-house validation processes are an important source of error, this paper presents a detailed procedure for in-house method validation based on simple experimental design and consistent statistics. The minimum performance criteria, based on "fitness for purpose" principle were defined and experimentally determined in order to demonstrate that the method is suitable to solve the particular analytical problem. The established criteria for performance characteristics such as selectivity, limit of detection and quantification, linear and working range, trueness, precision and ruggedness form the basis of the final acceptability of analytical data and of the validated method. The purpose of the proposed method is to monitor the process of catalytic wet air oxidation of bisphenol A in landfill leachates by using high performance liqui chromatography in order to study the influence of various parameters such as different kinds of catalysts, reactor loadings and operating conditions as well as to enable kinetic and mechanistic dissemination.

BPA separation and quantification was done by HPLC with UV detection (10 mm optical path) and chromatograms were extracted at 210 nm. A HP Agilent 1100 system, composed of degasser (type G1322A), isocratic pump, ALS autosampler (type G1329A), consisting of diode array detector (type G1315A) and ChemStation software was used. The mobile phase methanol (HPLC grade) and water (volumetric ratio 75:25) was filtered through a 0.45 µm PTFE membrane filter and degassed in an ultrasonic bath prior to use. The column used was Phenomenaex Luna 5u C18(2) column (250 × 4.6 mm, 5 µm) thermostated at 30° C. Flow rate of the mobile phase was 1.0 mL min^{-1} with sample injection volume of 20 µL and analysis run time of 9 min. BPA was identified using the HPLC system software by comparing retention time and UV absorption with those of reference solutions.

The key criteria for evaluation of an analytical method were the following: working range and linearity, limit of detection, limit of quantification, selectivity, trueness, precision (repeatability, reproducibility) and ruggedness of the method (Fig. 1). Measurement uncertainty of mass concentration of bisphenol A was evaluated based on method validation data (the "top down approach"), assuming that they comprise the total analytical procedure. All the contributions were obtained from the statistical analysis of repeated measurements to estimate the combined uncertainty. Trueness of the method was calculated from analyte recovery from spiked samples. Calibration with pure substance reference materials delivered appropriate results for ground water samples; on the contrary for landfill leachate matrix match calibration was required to enhance the trueness. Repeatability and within-laboratory reproducibility were evaluated from precision experiments. After estimation of all sources of uncertainty they were combined according to law of propagation of uncertainties, obtaining the combined standard uncertainty ($u(C_{BPA})$). The final result was reported as an expanded uncertainty ($U(C_{IMS})$), calculated as $U(C_{BPA}) = k \cdot u(C_{BPA})$, where k is the coverage factor.

When the distributions of the various uncertainty components are normal, a value of *k* of 2 roughly corresponds to a 95% confidence level. Validation results are presented in Table 1.

Validation Scheme

Working and linear range

- Working and linear range is the range of analyte mass concentrations over which the method can be applied. It is necessary to determine the range for any quantitative method.
- At the lower end of the mass concentration range the limiting factors are the values of the limits of detection and quantification, while at the upper end limitations are imposed by various effects depending on the instrument response system.

Limit of detection and limit of quantification

- Limit of detection (*LoD*) is an indication of the level at which detection becomes problematic. Limit of quantification (*LoQ*) is the lowest mass concentration of the analyte that can be determined with an acceptable level of repeatability precision and trueness (repeatability lower than 10 %, recovery between 90 and 110 %).
- Limits of detection and quantification were determined on the basis of signal-to-noise ratio required to be higher than 3:1 and 10:1, respectively.

Selectivity

- Selectivity is the degree to which a method can quantify the analyte accurately in the presence of interferences under the stated conditions of the assay for the sample matrix being studied.
- This was done by comparing the slope of the calibration curve obtained with reference solutions with the slope of the calibration curve obtained with reference solutions in real matrix samples.
- Matrix-match calibration curve was introduced to enhance trueness.

Trueness

- Trueness of the whole analytical procedure was tested through determination of recovery of the spiked samples of the selected relevant matrices. Ground water and landfill leachates were spiked on ten concentration levels and recovery *R* was calculated as $C_{obtained}/C_{spiked}$ (expressed in %).
- The method was selective in case of matrix-match calibration.
- The trueness of the method was proven with significance tests, when recovery of the method was not significantly different from one with threshold of 1 % as statistical significance.

Precision

- The most common measurements of precision are repeatability and reproducibility,
- For the estimation of repeatability, water samples of different matrices (ground water, landfill leachates) were spiked at ten different concentration levels.
- Within-laboratory reproducibility was determined by analyzing bisphenol A solutions (5.00 mg L^{-1}, 10.00 mg L^{-1}, 15.00 mg L^{-1}) in different days in the period of three months using different equipment and analysts at various environmental conditions.

Ruggedness

- To deal with eventual equipment failure, a study was conducted using bisphenol A reference solution (10.00 mg L^{-1}).
- Stability was evaluated after 24 and 48 h at different conditions: 25°C (room temperature) in the dark and at 4°C (refrigerator temperature) in the dark. Recovery between peak area of reference solution, stored at the defined environmental conditions and storage time with regard to the freshly prepared reference solution was calculated and the student`s test was used to evaluate significant difference from one (1 % level of risk).

Figure 1. Scheme for validation of the method for the determination of mass concentration of bisphenol A in landfill leachates.

Table 1. Validation parameters of the method for determination of bisphenol A in landfill leachates by using High Performance Liquid Chromatography.

Validation parameter	*Result*
Selectivity	*No eluting peaks at retention time of bisphenol A elution*
Limit of detection	*0.062 mg L^{-1}*
Limit of quantification	*0.125 mg L^{-1}*
Linear range	*2.0 – 20.0 mg L^{-1}*
Accuracy (recovery)	*97.0 – 106.0 %*
Precision	
- Repeatability	*3.0 %*
- Reproducibility	*3.2 %*

Our analytical protocol allowed us to quantify mass concentration of bisphenol A in landfill leachates between 2.00 and 20.00 mg L^{-1} with satisfactory recoveries (97.0 – 106.0 %) and repeatability lower or equal than 3.0 %. We also estimated within-laboratory reproducibility over three months period at various concentration levels which was found to be under 3.2 % in all cases. Measurement uncertainty of results was evaluated to be 10.2 % with 95% confidence level. Finally, the results revealed that the method is suitable for efficient monitoring of BPA mass concentrations in landfill leachates.

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POSTER PRESENTATIONS

DISTRIBUTION OF Cu, Fe AND Cd AMONG SE-HPLC SEPARATED CYTOSOLIC PROTEINS ISOLATED FROM LIVER, GILLS AND INTESTINAL PARASITE OF EUROPEAN CHUB (*Squalius cephalus* L.)

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Metal contamination in freshwater ecosystem is a critical environmental issue due to metal uptake, accumulation and possible toxicity in many aquatic organisms. Fish are one of the most indicative species in freshwaters, for the estimation of trace metal pollution and possible risk to human health. The pattern of metal distribution in fish depends on ambient metal concentration, its speciation and the uptake routes, which involve dietborne (intestine) and waterborne (gills) metal exposure, followed by blood transfer to target organs for metal storage and detoxification (liver and kidney). Trace metal detoxification processes depend mainly on metal binding to metallothioneins, inducible cellular proteins which reduce toxic metal effects (Hg, Cd, Ag) and take part in homeostasis of essential metals (Zn, Cu). To elucidate the metabolism, bioavailability and toxicity of metals in the aquatic environment, it is not sufficient to determine total tissue or cytosolic metal concentrations, since metals in living organisms form part of bigger, more complicated molecules, mainly proteins. So far, biological functions and mechanisms of metal toxicity in different organisms are still not thoroughly investigated, and the proteins to which they bind are only partially identified. In addition to already established assessment of metal exposure, by analysing metal and metallothionein concentrations in fish indicator organs, investigation of metal subcellular partitioning, involving proteins other than metallothioneins, could provide a more complete understanding of potential mechanisms of metal toxicity. Moreover, during the last few decades it is discovered that fish intestinal parasites (class Acanthocephala) effectively accumulate metals, especially toxic ones. Although metal concentrations in parasites highly exceed those in fish tissues, there is no information on metal distribution or detoxification in acanthocephalans.

The aim of this study was to compare the distribution of essential (Cu, Fe) and non-essential metals (Cd) among cytosolic protein fractions of gills (metal uptake site), liver (metal detoxification site) and acanthocephalans (*Pomphorhynchus laevis)* of European chub from the Sutla River, Croatia. The fractionation of cytosols was carried out by size exclusion high performance liquid chromatography (SE-HPLC; Superdex™ 200 10/300 GL column), and metal measurements in collected chromatographic fractions were performed off-line by high resolution inductively coupled plasma mass spectrometry (HR ICP-MS). In conditions of low metal contaminated river water, basic distributions of analysed metals indicated metal association with specific proteins, for Cu and Cd with metal binding protein, metallothionein and for Fe with Fe-storage protein, ferritin. The obtained information within this study presents the starting point for identification and characterization of specific metal-binding proteins in chub and acanthocephalans, which could be further on used as markers of metal exposure or effect on fish.

DETERMINATION OF TRANS FATTY ACIDS IN FOODSTUFFS BY GC/MS AND GC/FID

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Trans fatty acids (TFA) can enter the human body with food. Trans fatty acids have negative impact on the human health and therefore it is recomended to reduce their intake. Naturally they are in the milk and adipose tissue of ruminants, but much larger sources of these compounds are in hydrogenated vegetable oils. Hydrogenated vegetable oils are widely used in industry, fast food, semi-finished foods, fried foods, bakery products and confectionery products. In Europen Union except for Denmark, TFA levels are required on labels only if a TFA claim is made (e.g. ´low in TFA´). In 2003 the Danish Nutrition Council recommended restrictions of the use of TFA in foods, < 5% TFA were permitted in oils and fats used in processed foods.

During 2011 in SP laboratory we have analyzed composition of the trans fatty acids in about 74 samples of vegetable oils and fats and 178 samples of other types of foods (margarine, bakery and confectionery products, semi-finished foods, fried foods). Special attention is paid to the major trans isomers of fatty acids C18:1 and C18:2. Samples were prepared by standard method ISO 5509[1] and determined by standard method ISO 5508[2].

The fatty acid methyl esters (FAME) were analysed by gas chromatography using Agilent 6890 gas chromatograph with a very high column polarity (Agilent GC capillary column H88; 100 m x 0.25 mm i.d., film thichness: 0.20 μm), Mass Spectrometry Detector (5972A, Agilent) and Flame Ionization Detector. Peak identification of FAME was verified by mass spectrometry and compared with standards (Supelco 37 Comp. FAME Mix; Supelco,USA) .

Results are expressed as percentage of all fatty acids detected with a chain length between 8 and 24 carbon atoms.

In 51.3 % of fats and oils samples and 65.4% of other types of foods TFA level was less than 0.02%. For 42.7% of fats and oils samples and 20.7% of samples of other types of foods TFA level was between 0.02% and 5% while in 6% of fats and oils samples and 13.9% of other types of foods TFA level was greater than 5%.

Reference:

1. ISO 5509 Animal and vegetable fats and oils - Preparation of methyl esters of fatty acids.
2. ISO 5508 Animal and vegetable fats and oils - Analysis by gas chromatography of methyl esters of fatty acids

DETERMINATION OF ANTHOCYANINS IN HYBRID WINE BY HPLC-DAD AND HPLC-MS

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Anthocyanins are a family of polyphenols that are directly responsible for colour in grapes and wines. The anthocyanins profile of a grape and its wine, determined by the relations of the different anthocyanins, is characteristic of each variety. The hybrid grapes include anthocyanins monoglucosides and diglucosides. The objective of this work is to determinate and identify anthocyanin profile in hybride wines from Cabernet Cortis, vintage 2009.

Wine samples were directly injected to HPLC with diode array detector. Identification was carried out by comparing spectra and retention times with standards. The eluation order was confirmed by HPLC-MS analysis. In this experiment reversed phase column (Luna Phenyl-Hexyl, 250 x 4,6 mm, i.d. 5 μm, Phenomenex, USA) with gradient eluation and UV detection at 518 nm was used. The solvents were (A) aqueous 0,1% formic acid, and (B) acetonitril containing 0,1% formic acid. Mass spectrometry (MS) was done by electrospray ionisation in positive ion mode. Obtain mass spectras were compared with this one find in the literature. (Favretto, Flamini, Am. J. Enol. Vitic. **51** (1) 2000.) This analysis confirm eluation order obtain by previously describe method.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS OF *S*-ADENOSYLMETHIONINE AND *S*-ADENOSYLHOMOCYSTEINE IN FIBROBLASTS WITH *S*-ADENOSYLHOMOCYSTEINE HYDROLASE DEFICIENCY

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S-adenosylhomocysteine hydrolase deficiency (SAHH) is a rare disease, described for the first time in 2004. SAHH is one of the enzymes in methionine cycle, in which it catalyzes the hydrolysis of *S*-adenosyhomocysteine (AdoHcy) to adenosine (Ado) and homocysteine (Hcy). AdoHcy is formed by transmethylation processes in which CH3 group is transfered from *S*-adenosylmethionine (AdoMet) to numerous methyl acceptor molecules using methyltransferases (MT). Ratio of AdoMet/AdoHcy concentration is called methylation index of the cell. Methylation index is considered to be one of the key players in disease development by inhibiting metyltransferases and leading to misregulated methylation of molecules within cell. The purpose of this work is to determine methylation index in fibroblast cell culture with *S*-adenosylhomocysteine hydrolase deficiency.

Fibroblasts cultivated in 75 cm2 flasks were washed in phosphate buffer solution. Immediately after washing, 5 ml of 0.6 N perchloric acid with N-6-methyladenosine as an internal standard was added to cells. Cell debris was collected into tubes by scraping. The tubes were shaken thoroughly, placed on ice and centrifuged (4 000 x g, 10 min at +4 °C). The supernatant was adjusted to pH 5.5 by adding 2 M K_2CO_3/1 M KH_2PO_4 and then centrifuged. Supernatant was applied onto solid-phase extraction column (BondElut LRC-PBA). Elution of AdoHcy, AdoMet and internal standard was performed by using 0.1 M HCl and the eluate was analysed by high performance liquid chromatography (MN CC 250/4.6 NUCLEODUR C18 Gravity 5 μm) with UV-detection (258 nm). The mobile phase consisted of solvent A (50 mM natrium dihydrogenphosphate, 8 mM heptanesulfonic acid sodium salt with addition of o-phosphoric acid, pH 3.0) and solvent B (methanol).

High performance liquid chromatography was previously used for AdoMet/AdoHcy ratio analysis in whole blood. Here, we demonstrate that AdoMet/AdoHcy ratio can be measured not only in whole blood, but also in cells, which could be useful biomarker at cellular level for the diagnosis of *S*-adenosylhomocysteine hydrolase deficiency. Our results suggest that the AdoMet/AdoHcy ratio is significantly lower in SAHH deficient fibroblast cells when compared to control.

DATA QUALITY IN WATER ANALYSIS: VALIDATION OF A METHOD FOR THE DETERMINATION OF ANIONS IN WASTE WATER BY USING ION CHROMATOGRAPHY

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Ion chromatography provides a simple, fast, small sample volume demanding, and fit-for-purpose methodology for the concurrent determination of anions in waste water. Current law legislations, as well as the need to constantly provide accurate and reliable results, enforce laboratories to practice quality management system, according to the requirements of the ISO/IEC 17025 standard. In this paper, the work that was undertaken for the accreditation of ion chromatographic method for determination of selected anions in waste water, is presented.

The laboratory has developed in-house method, which is based on the standard ISO 10304-1 (2010). The instrumental setup used was an automated ion chromatograph equipped with chemical suppression system and conductivity detector for anion analysis. Deviations from the standard procedure made a full method validation imperative, therefore, for all anions (chloride, nitrite, nitrate, sulfate), method characteristics were determined and assessed towards the fitness-for-purpose scope (range of measurement, calibration, method detection level, level of quantification, repeatability, reproducibility, accuracy, peak resolution). Additionally, measurement uncertainty was determined following the »top down« approach. The present validation fits to the repeatability and reproducibility limits imposed by the water legislation. The analysis uncertainty increases at lower concentration levels and decreases at medium and high, where the relative expanded uncertainty is lower than 6 % for all the analytes. At concentration levels near the quantification limit of the method, the uncertainty obviously increases, but even in these cases, the results are satisfactory. Procedures for daily measurement quality control and regular participation in proficiency testing schemes were also implemented.

Methodology and results for method validation, uncertainty quantification and quality control is presented in detail. The significance of this paper falls on the statistical study of the analytical method, where all the uncertainty contributions involved in the process have been considered.

APPLICATION OF HYPHENATED ANALYTICAL TECHNIQUES FOR THE ASSESSMENT OF ENVIRONMENTAL METAL EXPOSURE

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Depending on their concentration non essential but also essential trace metals and metalloids can cause toxic effects on living organisms. Determination of the total content of selected metals in biological tissue is regularly implemented in environmental monitoring programs, but this is insufficient for the metal exposure assessment. On the other hand, the early effects of metal contamination can be already observed at the molecular and cellular level of an organism chronically exposed to even low doses and in this way may serve as an "early warning" indicator.

Response of an organism to metal charge can be achieved by binding metals to various cytosolic biomolecules as ligands. Therefore, the investigation of cytosolic profiles of metals bound to biomolecules of different molecular size can be of great importance as a first step in defining possible biomarkers of metal exposure in the field of pollution monitoring. Coupling size exclusion high performance liquid chromatography (SE-HPLC) with online detection by a sensitive and multi element isotope detector such as an ICP-MS represents an excellent combination which enables obtaining such information.

In this contribution cytosolic patterns of metals associated with biomolecules of different molecular size present in the digestive gland of wild mussels (*Mytilus galloprovincialis*) as a common bioindicator organism were studied. Mussels' cytosol-protein profiles from several marinas and harbours considered as fairly polluted areas and reference location were compared. Size exclusion chromatography was performed at Superdex 75 (molecular weight range: 3 kDa – 70000 kDa) and Superdex 200 (molecular weight range 10 kDa – 600 kDa) in order to obtain profiles for selected metals bound to a wide range of molecular size biomolecules.

Metal profiles from more polluted locations have indicated increased intensities in chromatographic fractions related to specific biomolecule size ranges, which are characteristic for selected known metal containing biomarkers for that specific metal (such as metallothionein for Cu, Zn and/or Cd). Preparative chromatography of these fractions can be a starting point for subsequent purification and identification of proteins with the final goal of defining a set of biomarkers specific for metal pollution.

This study was performed within the scope of the DAAD project (Project-ID 50752021 Deutscher Akademischer Austausch Dienst) during which scientific collaboration between the research groups from Croatia and Germany has been established.

IMPACT OF ADHESIVE TAPES AND EXTRACTION METHODS IN THE DETERMINATION OF NATURAL MOISTURIZING FACTORS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Stratum corneum (*SC*) is the outermost part of the skin and has important role in body protection against penetration of chemicals and pathogens, UV radiation and keeps safe the water balance in and out of the body. Filaggrin (filament-aggregating protein) plays a major role in *SC* structural integrity and is proteoliticaly degraded into a pool of free amino acids and their metabolites (histidine, trans- and cis- urocanic acid, 2-pyrrolidone-5-carboxylic acid and tyrosine,) known as natural moisturizing factors (NMFs). NMF constituents are hygroscopic and responsible for maintaining hydration of the *SC*, pH of the skin, bacterial defense etc. Atopic dermatitis, a common skin inflammatory disease is associated with reduced level of NMFs and thus determination of NMFs in *SC* is of great interest.

Tape stripping is a simple and non-invasive method for sampling of the *SC in vivo*. In the present work we evaluated the performance of two different adhesive tapes to sample SC. Furthermore, we investigated two different methods for extraction of NMF components from the tape strips.

We investigated two commercially available tape strips: D-Squame and Corneofix. Extractions of NMFs from these tape strips have been performed by using ammonia and potassium hydroxyde. NMFs recovered from the tape strips were analyzed by reversed phase high performance liquid chromatography. To normalize for the amount of the *SC* harvested by the tape, we determined the amount of proteins on the tape by measuring 1) optical density and 2) colorimetric method (Pierce assay). This investigation provided more insight in the factors that influence performance of the method used for the determination of NMFs in the *SC*.

***Keywords*:** natural moisturizing factor, tape stripping, stratum corneum, atopic dermatitis

IDENTIFICATION AND QUANTIFICATION OF PLANT HORMONES AUXINS IN BRASSICA RAPA SEEDLINGS USING GC-MS

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Plant hormones (phytohormones) play an important role in regulating the growth, metabolism and development of plants. Among them auxin indole-3-acetic acid (IAA) has been firstly identified and studied since Darwin's time. In plants, auxins occur in a very low amount. Therefore, research in the field of biochemical processes in which auxins are involved includes application of highly sensitive analytical methods for their identification and quantification.

In research presented here we used GC-MS (gas chromatography–mass spectrometry) method for identification and quantification of IAA, indol-3-propionic (IPA) and indole-3-butyric (IBA) acids in *Brassica rapa* seedlings upon treatments with 100 mM of jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA) in comparison to controls. For auxin identification samples were prepared by liquid/liquid extraction followed by solid-phase extraction (SPE), after which samples were methylated using diazomethane.

In a full scan mode that was used for auxin identification we found IAA in all samples, while occurrence of IBA and IPA were only sporadically determined and could not be reproducible. Quantification of IAA was done in μSIS mode because of its higher sensitivity, by adding [$^{13}C_6$] IAA as internal standard. In addition to free IAA, total IAA was determined after alkaline hydrolysis with 7N NaOH at 100 °C for 3 h. We found significantly higher amount of free and conjugated IAA in samples treated with jasmonic acid (free: 11.10±1.55 ng/mg dw; conjugated: 126.62±3.90.12 ng/mg dw) in comparison to control (free: 4.63±0.21 ng/mg dw; conjugated: 43.47±5.54 ng/mg dw) samples. Treatments with SA and ABA did not significantly influence IAA content in comparison to controls. Sporadically identification of long-chained auxins IPA and IBA confirmed their potential occurrence in *B. rapa* plants but detailed protocol for identification needs to be established.

DEVELOPMENT AND VALIDATION OF LC–MS/MS METHOD FOR QUANTIFICATION OF OPIORPHIN IN HUMAN SALIVA

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Opiorphin, QRFSR-peptide (Fig.1), is a mature product of the PROL1 (proline rich, lacrimal 1) protein that showed beneficial effects in pain management, antidepressant-like actions as well as involvement in colonic motility and erectile physiology. Using opiorphin as a potential biomarker of different pathological states requires the development of robust and sensitive methods. We developed a highly sensitive and specific liquid chromatography with tandem mass spectrometric detection (LC–ESI–MS/MS) analytical method for the analysis of opiorphin in human saliva with limited sample manipulation[1]. Quantification was based on multiple reaction monitoring using characteristic transitions (*m/z* 347/120 – as quantifying ion; 347/175 and 347/268 as qualifying ions).

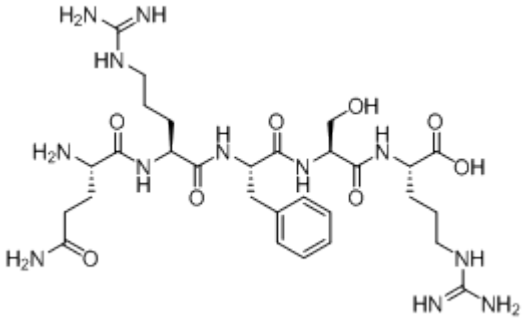

Figure 1. Molecular structure of opiorphin

The analytical method for determination of opiorphin in the human saliva was also validated on the following parameters: linearity, limit of detection (LOD), lower limit of quantification (LLOQ), precision, accuracy, recovery, matrix effect and sample stability. The assay was linear in the range of 0–110 ng/ml and the lower limit of quantification reached was 1.0 ng/ml. The intra-day precision and accuracy were between 2.7–5.6% and−2.3 to 3.2% , respectively. The inter-day precision and accuracy were between 10.8–13.7% and−11.0 to 52%, respectively. Mean recovery was 106% and mean matrix effect was 0.97. Opiorphin in TFA treated saliva samples was stable for at least 12 h at room temperature and up to 30 days at −20 °C. Opiorphin levels in human saliva samples collected from young healthy individuals ranged from 2.8 to 25.9 ng/ml.

1. L. Brkljačić, M. Sabalić, I. Salarić, I. Jerić, I. Alajbeg, I. Nemet, Development and validation of a liquid chromatography – tandem mass spectrometry method for the quantification of opiorphin in human saliva, *J. Chromatograph. B* 879 (2011) 3920 – 3926.

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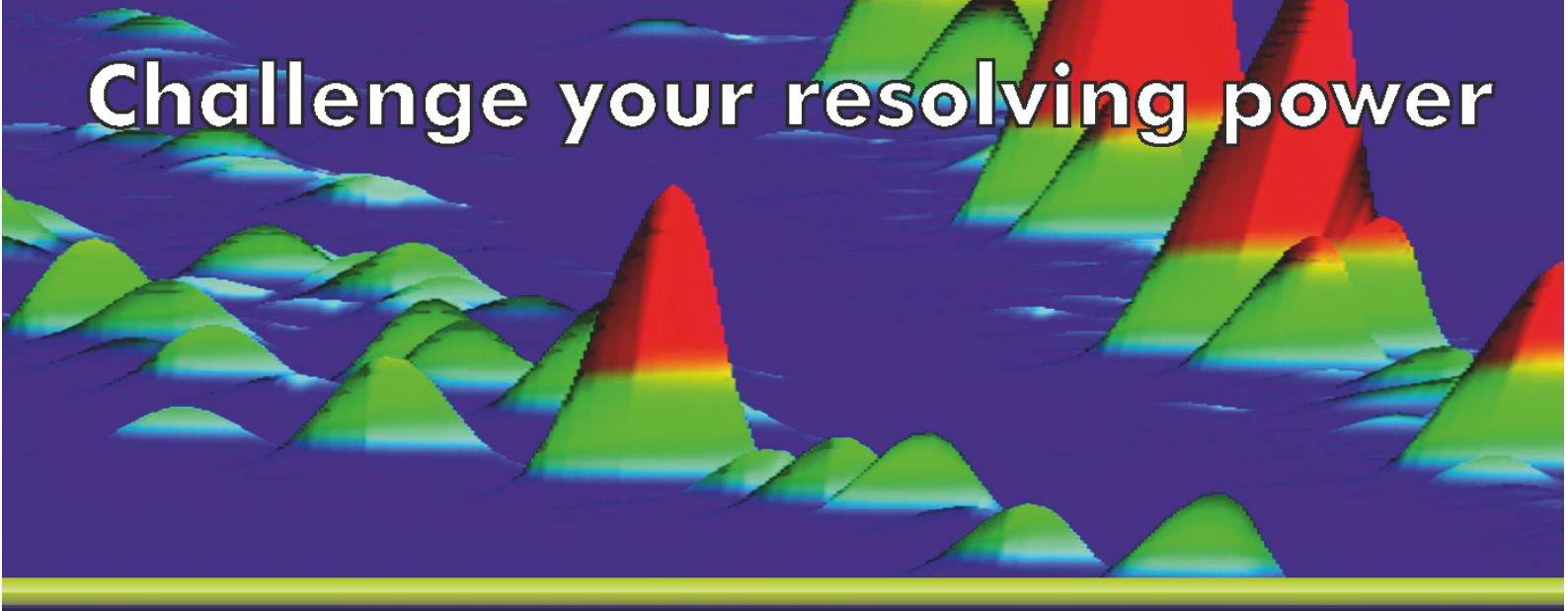

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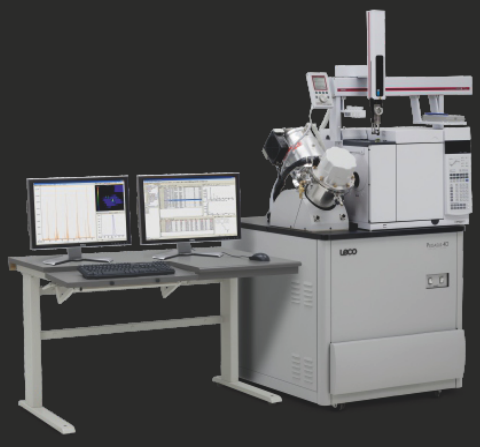

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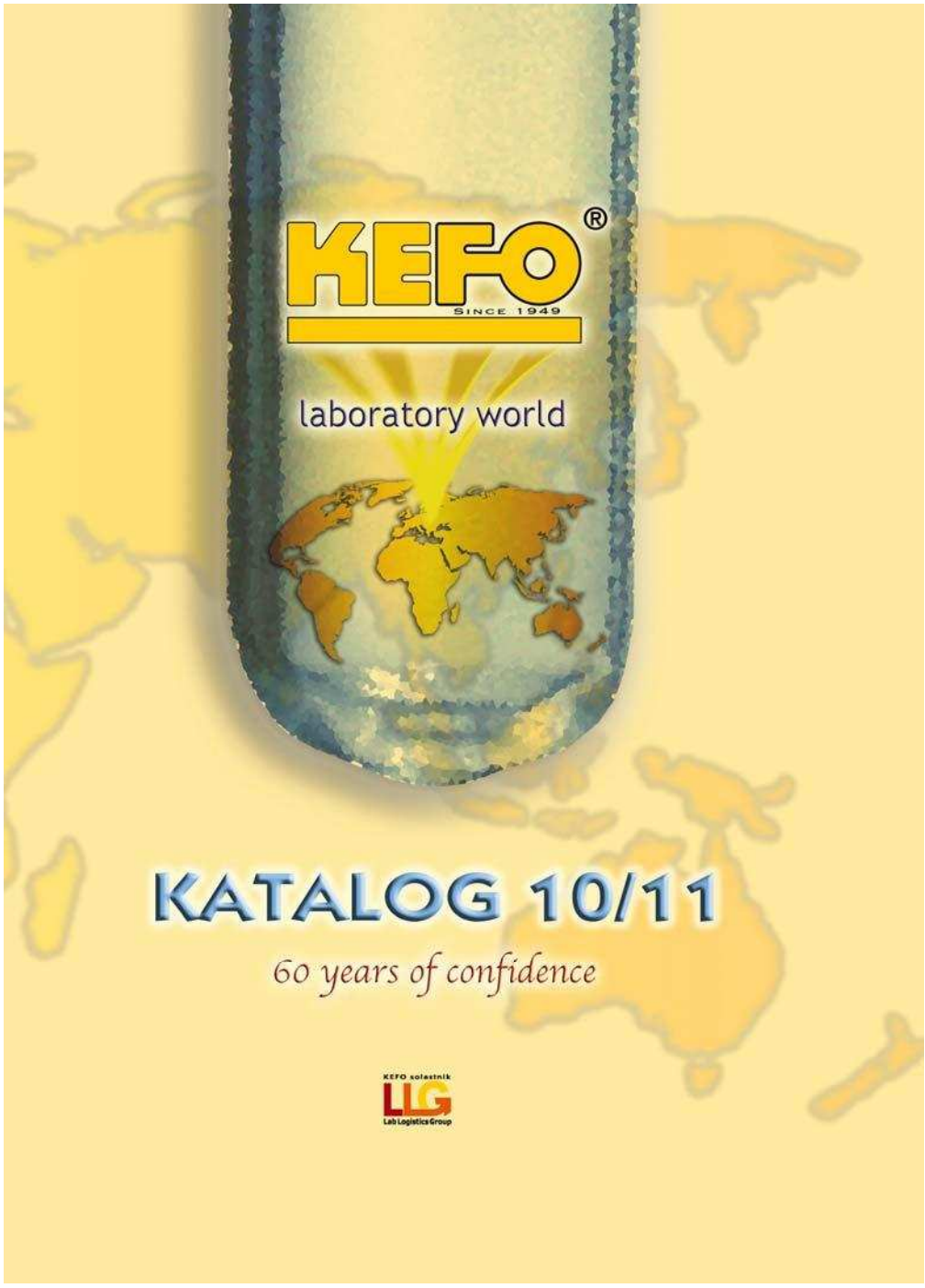

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