Review

Overview of the Development and Application of the Hyphenated Techniques in Nutritional Analysis

Darja Kotnik,[1] Andrej Šmidovnik,[1,2]* Petra Jazbec-Križman,[1] Mitja Križman[1] and Mirko Prošek[1]

[1] *National Institute of Chemistry, Laboratory for Food Chemistry, Hajdrihova 19, SI-1000 Ljubljana, Slovenia*

[2] *EN-FIST Centre of Excellence, Dunajska c. 156, SI-1000 Ljubljana, Slovenia*

** Corresponding author: E-mail: andrej.smidovnik@ki.si*
Tel. ++386 1 47 60395; Fax: ++386 1 47 60300

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Abstract

The development of some sensitive assays for quantitative nutritional analysis with an emphasis on selected hyphenated analytical techniques is reviewed in the present paper. The majority of work is dedicated to reviewing the development of analytical tools for routine analysis of carbohydrates and lipids in biological samples, many of them introduced in our laboratory. Handling biological matrices, where endogenous compounds can mask the analyte of interest or where the occurrence of the coelution effect of several compounds present in different amounts hinders the analyte's peak integration, is a major challenge. To overcome this challenge, hyphenated techniques have become widespread in laboratory practice. Some of these techniques are reviewed, with special attention given to an effective on-line interface for thin-layer chromatography-mass spectrometry and on-line coupling thin-layer chromatography-gas chromatography. Recently introduced an on-line coupling of ion chromatograph and hybrid RF/DC quadrupole-linear ion trap mass spectrometer represent an analytical tool for the solution of bioanalytical problems. Developed methods using ion chromatography-pulsed amperometric detection and ion chromatography-mass spectrometry techniques for the quantitative evaluation of sugars are presented. This paper represents basic contributions of our research work connected with some of modern hyphenated techniques. However, this review is restricted to the published papers to be significant developments or improvements during the last three decades.

Keywords: Carbohydrate and lipid analysis, hyphenated techniques, ion chromatography-mass spectrometry, thin-layer chromatography-mass spectrometry, thin-layer chromatography-gas chromatography

1. Introduction

Nutrition has a decisive effect on our health and diet helps us to keep the proper amount of necessary nutrients in our organism's blood and cells. Healthy food contains appropriate amounts of lipids, carbohydrates, proteins, vitamins, antioxidants, coenzymes and enzymes. However, an excess of free radicals or a lack of antioxidant defense can damage all the constituents of human body. The body's antioxidant defense network confines the negative effects of free radicals and thus prevents oxidative damage. This network consists of large molecules of antioxidant enzymes and coenzymes, together with small molecules of micronutrients. Many papers describe the higher incidence of almost every degenerative disease with decreased levels of vitamin E, vitamin C, carotenoids, coenzyme Q_{10}, bilirubin, alpha lipoic acid, glutathione and other elements, such as selenium. Thus, in order to verify the functioning of the body's antioxidant defense system, the blood serum levels of many active small-molecules responsible for antioxidant defense has to be monitored. Our laboratory is focused on such research.[1–5]

The growing interest in the biological role of nutrients has led to incessant development of analytical methodology for their detection. Due to the complexity of the matrix in natural samples, the separation of analytes can be hindered by coelution effects, as well as excessive noise and inappropriate peak attribution and integration induced by interferences. Mass spectrometry (MS) is a highly sensitive and selective analytical tool and various types of

ionization devices and mass detectors have been developed over the past 50 years: from high-resolution magnet detectors, triple stage quadrupole detectors with MS/MS capability, ion trap detectors with MS^n capability, TOF (time of flight) detectors to Fourier transform MS detectors and orbitrap detectors. This continual development in the field of mass spectrometry has been reviewed by many authors.[6–10] The analysis of complex mixtures, such as extracts of food products and bodily fluids to study metabolic pathways, requires highly selective analytical techniques to identify and quantify targeted components and to characterize unknown compounds. Hyphenated techniques, which combine chromatographic and spectral methods to exploit the advantages of both, are thus preferable in modern analytical chemistry. On-line coupling of these two techniques offers many advantages such as higher sample throughput and thus shorter analysis time, better reproducibility and selectivity, and reduced contamination due to the closed system and additional information.

Therefore, some new promising combinations of chromatographic systems with spectrometric detection have appeared. In this context, a noticeable contribution is the simple but effective on-line interface for thin-layer chromatography-mass spectrometry (TLC-MS).[11,12] The schematics of the TLC-MS interface is illustrated in Figure 1 and a scanning TLC-MS of cholesterol is shown in Figure 2. Up to now, the off-line operation has mostly been used. Separated spots were scrapped from the plate and substances were eluted from the sorbent and injected into MS. This off-line operation is achieved without any special equipment and extra cost, but it is time consuming. More convenient on-line extraction has been invented, and different working prototypes have been prepared and used, one of which was created in our laboratory. The commercially available instrument incorporates the above-described principles and enables elution of spots and direct transfer into the ionization device and MS detector. It is expected that this instrument will have an immense influence on the development of new analytical procedures.

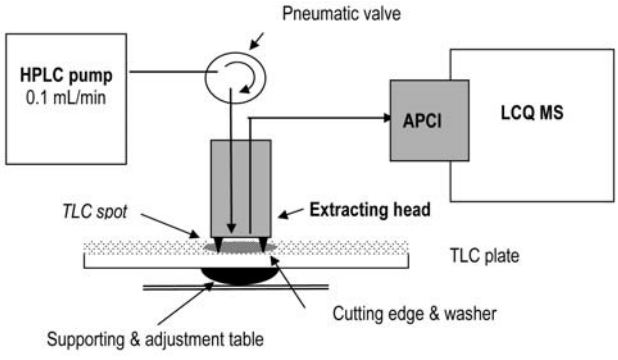

Figure 1. Schematics of TLC-MS interface with the extraction head. The cutting edge, which penetrates into TLC layer and prevents leaking, is shown. A specially shaped supporting table prevents breaking of the TLC plate. Compressed air at 5 bars keeps the extraction head on the TLC plate.

A second important achievement is a new type of hybrid RF/DC quadrupole-linear ion trap mass spectrometer coupled to ion chromatograph (IC-MS/MS), which has recently appeared on the market. Up to now, IC was not considered as a suitable technique for combination with MS. The huge amount of ions arising from the separation process produces a vast uncontrolled background noise. In spite of this, some of our researchers acquainted with modern trends in IC and purchased a new IC-MS/MS system from two established manufacturers, Dionex (Sunnyvale, CA, USA) and MDS/Sciex, (Concord, ON, Canada). Above mentioned mass spectrometer is based on a triple-quadrupole ion path, and is capable of all of the conventional tandem MS scan modes, and also several high-sensitivity ion trap MS scans, using the final quadrupole as a linear trap.

The present coupling of ion chromatography with tandem mass spectrometry (IC-MS/MS) covers a wide range of compounds determination. Presented example from carbohydrate analysis in serum samples is described to demonstrate the basic capabilities of the coupled IC-MS system. The choice of an interface for a particular application depends on the polarity and molecular mass of the analyte. A development of electrospray ionization (ESI) for effective introduction of polar compounds into mass spectrometer encouraged the development of LC-MS and LC-MS/MS analysis of underivatized carbohydrates.[13–15] In general, three major challenges are met in IC-MS coupling. (i) The non-volatile and (ii) the highly alkaline solvents used particularly in ion chromatography separation of carbohydrates, incompatible with MS, (iii) the ionization of non-volatile and/or thermally labile analytes. The problem of incompatibility of the mobile phase composition has been solved by the application of micromembrane suppressors.[13,14] Compatibility problem related to flow rate has been solved in various ways. Conventional (3–4.6 mm i.d.) microbore and microcapillary columns are compatible with the selected MS system, which can handle up to 2 mL/min flow without any flow splitting. However, the trends in development in IC-MS technology is towards the capillary IC in place of the traditional analytical- and micro-scale IC without the necessity for flow splitting and consequently the loss of sample.[27]

The third combination, recently introduced by our group, is a comprehensive TLCxGC set-up for lipid analysis as an alternative method to comprehensive HPLCxGC techniques.

2. Applications

2. 1. Carbohydrates

Quantitative measurement of carbohydrates and their derivatives is useful due to their important role in a

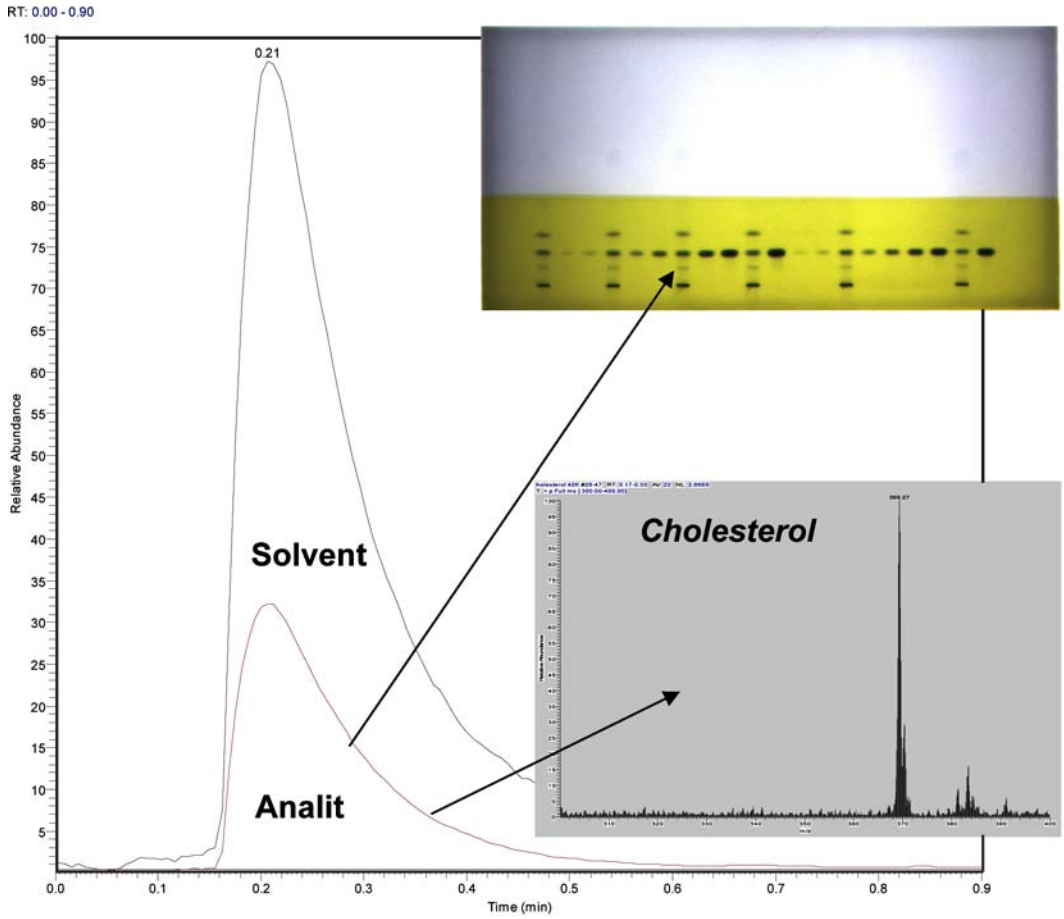

Figure 2. Results recorded with the TLC-MS system. Curves of the one selected spot eluted from the TLC plate and scanned with MS show a) total ion current and b) selected ion m/z = 369–370, corresponding to the spot of cholesterol.

wide range of biological processes. The increasing incidence of obesity and chronic diseases, such as diabetes mellitus, affect millions of people around the world. Oligosaccharides as parts of cell-surface glycoproteins play an important role in molecular recognition processes and determine a variety of the biochemical properties of these proteins. On the other hand, monosaccharides, such as glucose and galactose reflect, to a large extent, the health status of individual organism. The serum level of glucose is an important indicator and promoter of the oxidative states in plasma and may cause the progress of some degenerative diseases. The optimization of analytical tools is highly necessary (in terms of sensitivity, selectivity, accuracy and quick response) to improve the subsequent medical treatment of such diseases. Thus, carbohydrate metabolism research has led to a growing interest in the development of several techniques as powerful tools for carbohydrate evaluation. Analysis of carbohydrates and their metabolites in complex matrices requires very sensitive and selective techniques to discriminate between the peaks of interests and background noise.

Separation of carbohydrates is based on chromatographic methods, such as reversed-phase high-performance liquid chromatography (RP HPLC), TLC, IC and capillary electrophoresis (CE).[28–31] Detection is carried out using different spectrophotometric, conductometric, electrochemical, light scattering, and mass spectrometric detectors.

During a certain period, gas chromatoghraphy-mass spectrometry (GC-MS) was the technique of choice.[32–34] GC separation with FID or MS detection of carbohydrates requires a derivatization step with silylation, alkylation or acylation to convert the carbohydrates into adequate derivates, due to their high polarity, hydrophilicity and low volatility. Another convenient analytical technique is TLC. Carbohydrates are easily separated, derivatized and measured; but TLC is not sufficient to quantify samples with very complex matrices. When using the conventional detectors in LC analysis, there is always some vagueness associated with identification of target compounds and possible coeluted peaks. For UV or fluorescence detection preinjection or postcolumn chemical derivatization is needed to produce detectable analogues. Due to the absence of chromophoric or fluorescent groups in carbohydrates, a derivatization step is required. Labeling with 2-aminobenzamide provides carbohydrate

detection using a fluorescence detector at the picomole level.[35] Derivatization using 1-phenyl-3-methyl-5-pyrazolone (PMP) is preferred over pyridylamination (PA) owing to simplicity and enhanced ionization efficiency.[28] However, the time consuming nature of sample pretreatment including derivatization has necessitated the development of direct methods for carbohydrate determination.

Carbohydrates feature weak acidity with pK_a values between 12 and 14 and are at least partially ionized under strong basic conditions, depending on their pK_a values. Due to the extreme alkaline conditions (hydroxide solution as the mobile phase), only a strong polymeric anion exchange stationary phase can be used and thus high-performance anion-exchange chromatography (HPAEC) is the technique of choice for the separation of carbohydrates. However, the development of direct methods for carbohydrate determination has been hindered by the lack of suitable chromophore or electrophore groups, and hence more sophisticated methods have had to be adopted for carbohydrate analysis. Underivatized carbohydrates are commonly detected by refractive index (RI) detection,[36] which has several limitations, such as sensitivity to changes in solvent composition and gradient elution, temperature and pressure. The most widely used detection technique in carbohydrates analysis is electrochemical detection. Efficient methods to qualify and quantify carbohydrates in natural samples and food products are based on anion-exchange chromatography coupled with electrochemical detection at Au, and Pt working electrodes.[30,37–39] Electrochemical detection is one of the most sensitive and selective method, but measurements can be obstructed by the inactivation of the electrode surface by adsorbed oxidation products. To prevent this contamination process, pulsed amperometric detection (PAD) was introduced for cleaning and reactivation of the electrode surface.[40] In electrochemical detection of carbohydrates, a standard quadruple-potential waveform is applied to the gold working electrode, which exploits the electrocatalytic activity of carbohydrates at elevated pH values (greater than 12).[41] The generated current is proportional to the carbohydrate concentration, enabling sensitive detection and quantification. PAD coupled to HPAEC provides useful technique for routine analysis without time-consuming derivatization steps. As PAD is not selective it is necessary to use mass spectrometry for sugar identification. Therefore, IC-PAD is considered to be the most accurate routine method to qualify carbohydrates, whereas IC-MS provides a better selectivity for problematic samples and is the tool of choice to elucidate unknown peaks. MS detection is a promising technique, which includes some drawbacks. Selected carbohydrates can be isomers yielding identical mass spectra. Verification of the identity of individual carbohydrates is feasible using a comparison of chromatographic retention times with reference or isotope labeled compounds or with nuclear magnetic resonance (NMR) spectra.[42] MS/MS method represents an advanced quantitative analysis of complex carbohydrate mixture performed by creating the fragments of a molecular ion by collisionally activated dissociation (CAD) and thus providing fingerprint assays. Product ions are generated from the molecular ion by covalent bond cleavage within the carbohydrate backbone and/or losses of water. A very high specificity is obtained with a single ion monitoring channels, which allows the determination of coeluting compounds and the elimination of background from the non-carbohydrate components of the sample matrix as well as the mobile phase. A full scan channel is used to record mass spectra from unknown compounds.

Many strategies in LC-MS interfacing have been discovered for carbohydrate ionization.[43–46] The applicability of atmospheric pressure interfaces, such as atmospheric pressure chemical ionization (APCI) and ESI have led to further studies of carbohydrate ionization. Owing to the high polarity and low volatility of carbohydrates, electrospray ionization is preferred to APCI. However, the huge amount of ions arising from the IC separation process creates unwanted background noise and therefore this techniques has not been considered suitable for coupling to MS. Due to the high salinity and low volatility of the potassium/sodium hydroxide used as the eluent in the IC separation of carbohydrates, it can not be directly injected through the MS interface. High saline and low volatile hydroxide eluent can cause electrical shortcuts and capillary plugging by salt crystallization, which has to be removed prior to MS. Thus, the eluent and separated substances have to be sent through an anion self-regenerating suppressor to lower the pH value of the eluent to neutral levels. The use of a high-capacity suppressor (desalter) enables the coupling of IC and MS detection via an electrospray interface. Electrochemical desalting is an efficient way to convert hydroxide into pure water. The neutralization of KOH/NaOH is obtained by a combination of water electrolysis and ion-exchange: H^+ are produced by the oxidation of water, which are then exchanged with the K^+/Na^+ ions of the eluent, which is therefore neutralized without diluting. Background noise is significantly decreased and the resulting eluent is compatible with MS detection. However, at this stage the MS detection of carbohydrates becomes challenging, as carbohydrates at neutral pH cannot be directly ionized by ESI. Underivatized carbohydrates are poorly detectable in ESI or APCI due to their low ionization yields. Many authors described on-line LC-MS analysis of carbohydrates using different ionization methods for carbohydrates molecules in neutral media. HPAEC-ESI-MS/MS has been used for the analysis of a mixture of neutral and acidic sugars in bacterial whole cell hydrolysates.[47] ESI-MS analysis has been applied in various fields, such as carbohydrate determination in bodily fluids, tobacco products, soft drinks, and atmospheric aerosols.[42,48–51] It was found that carbohydrates

readily ionize from aqueous solutions as a non-adducted molecular ion $[M–H]^-$. To obtain sensitivity, the elimination of the highly ionic mobile phase prior to entry into the ESI ion source was carried out using on-line suppressor. In addition, it is also possible to form ions of carbohydrates using inorganic ions. To improve the ionization efficiency, carbohydrates have been analyzed by ESI in positive ion mode as metal ion adducts (including sodium,

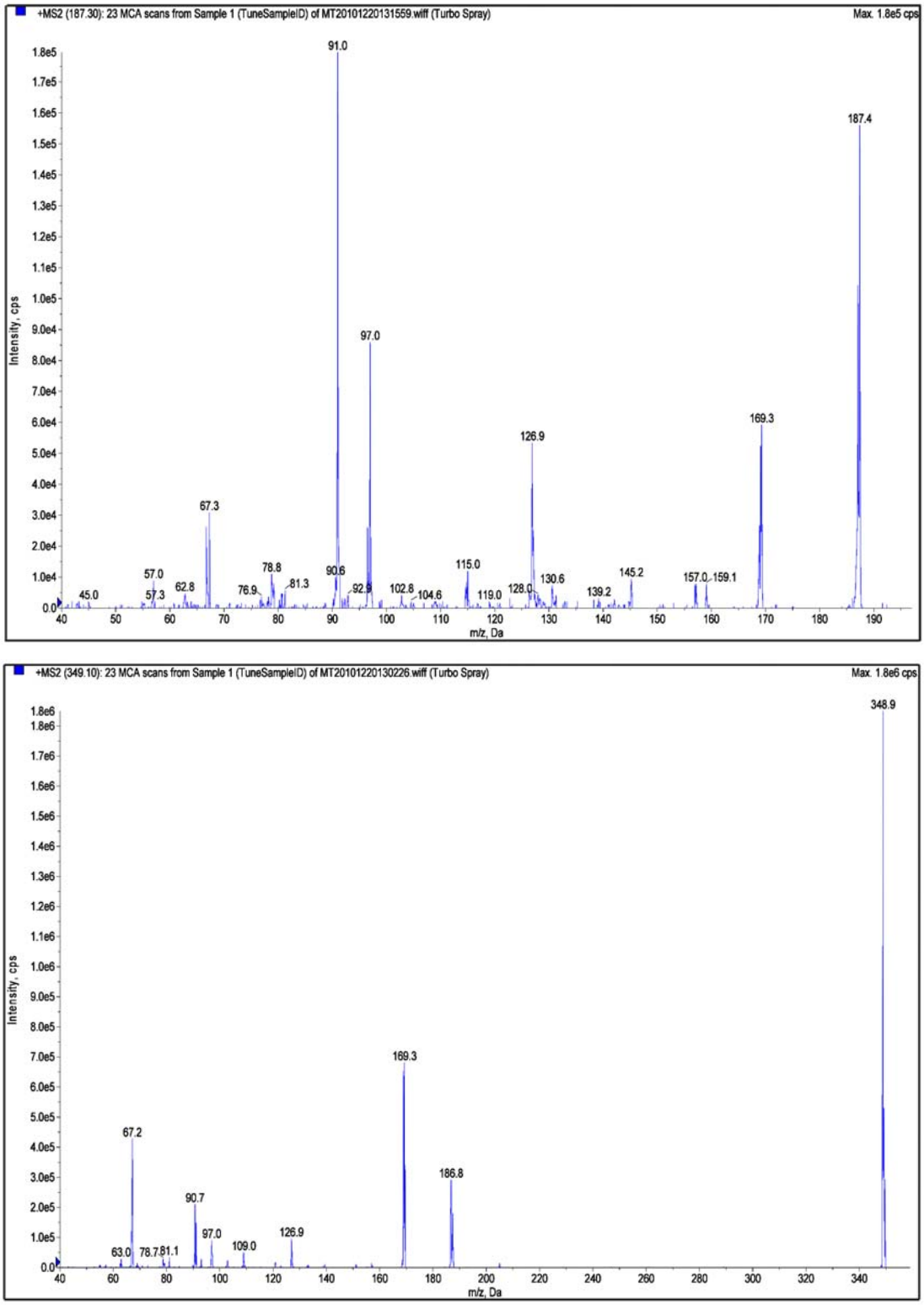

Figure 3. Typical mass spectra of glucose (above) and sucrose (below) as Li-adducts.

cobalt, lithium, cesium, lead) and in negative mode as anion adducts (including iodine chloride and acetate).[14,52–58] The post-suppressor addition of CH_3CN/H_2O solutions containing NH_4OAc or LiOAc have been used to provide low-nanomole detection of the monosaccharide by selected ion monitoring of the cationized ammonium adducts.[14,59] Regarding the literature, it was found that the most abundant carbohydrate metal adducts are formed with lithium adducts.[60,61] For that reason, a lithium chloride solution is continuously added in front of the ESI interface. Consequently, the response factor is greatly enhanced in positive mode by the formation of $[M+Li]^+$ ions. Typical mass spectra of glucose and sucrose as Li-adducts are represented at Figure 3. On the contrary, $[M+Cl]^-$ ions are poorly detectable in negative mode. An alternative method to post-column addition could be a partial suppression of the KOH/NaOH eluent in order to form adducts with the remaining K^+/Na^+ ions. As the suppressor current must be maintained at a constant value to avoid baseline drifting this method cannot be applied during gradient elution.

The purchase of the new IC-MS/MS instrument in Laboratory for Food Chemistry at the National Institute of Chemistry Slovenia enabled the development of routine analytical methods appropriate for samples with complicated matrices and low concentrations of analytes. The ion chromatography coupled to electrochemical detection and mass spectrometry (IC-ECD-MS/MS) system setup is shown in Figure 4. A described system was used for carbohydrate analysis on animal plasma samples. Samples were collected from chickens administrated CoQ_{10} and alpha lipoic acid. The synergistic effects of these two antioxidants were studied and we expected that the plasma glucose level would show us the oxidative status of the organism. Separated monosaccharides from blood were determined using MS/MS detection and electrochemical detection, where we compared the standard four-step and newly developed two-step potential waveform (unpublished results). Two-pulsed waveform in comparison to standard waveform eliminates the deleterious oxidative cleaning of adsorbed products. Newly developed two-step potential waveform will be described in details anywhere else. Due to the weak ionization of carbohydrates at neutral pH, a make-up solution (0.5 mmol/L LiCl) is added to create the ionic species for MS detection. Characteristic multi reaction monitoring (MRM) transitions of glucose (187.2/127.0) and sucrose (349.1/187.4) were used for carbohydrates monitoring in plasma samples. The amperometric detection cell and the MS detector were placed in parallel. The conductivity detector in front of the MS interface was set to monitor the suppression of high-value pH eluent.

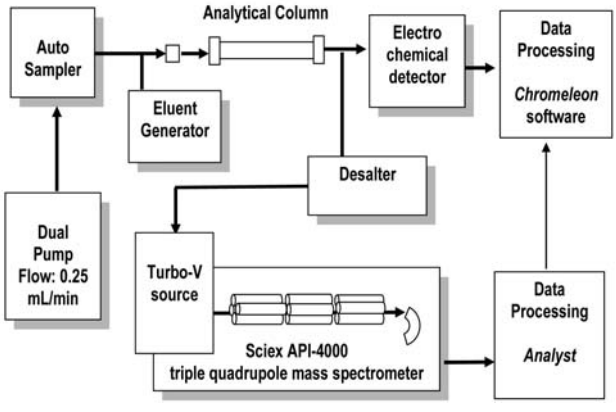

Figure 4. Schematics of the installed HPAEC-ECD-MS/MS system.

2. 2. Lipids

Lipids are one of the most complex groups of primary metabolites. Simple fatty acid profiling, usually done on their methyl esters (FAME) with GC, cannot provide information about fatty acid distribution/association within individual lipid groups (e.g. triacylglycerols, diacylglycerols and phospholipids), because such data are lost during sample preparation (transesterification). Thus, what we obtain from such a simple GC analysis is only an »average« fatty acid profile. The development of comprehensive HPLCxGC has become the technique of choice for such demanding analytical tasks.[62] However, HPLCxGC systems can be complicated in their management, due to the interfacing issues between HPLC and GC dictated by several experimental parameters (i.e. collection loop volume, FAME reaction rate, GC analysis time, etc.). Besides the instrumentation costs, setting up an HPLCxGC system can become very expensive. As an alternative method to HPLCxGC, a TLCxGC set-up for lipid analysis has been introduced in our laboratory. The interfacing between TLC and GC was accomplished by headspace sampling. Coupling TLC to GC provides more flexibility in terms of choosing the TLC chromatographic window width injected into the GC with fewer limitations compared to HPLC, where the collection loop volume dictates the chromatographic window width throughout the chromatographic run. However, the major issue in such a TLCxGC system is represented by the analyte vaporization rate. Thus, conditions for implementing the total vaporization technique (TVT) must be provided. New techniques have been tested for fingerprinting different edible oils with good results in terms of reproducibility and linearity. Lipid analysis with TLCxGC was performed on pumpkin seed oil . Pumpkin seed oil was diluted with ethyl acetate. An aliquot of sample solution was applied with Linomat (Camag) onto a 10 cm long silica gel, aluminum-backed TLC plate. The plate was developed with a mixture of petroleum ether-diisopropyl ether-acetic acid (80/20/1, v/v/v). Single sample tracks were cut into 1.2 cm wide strips. The strips were then chopped into 1 cm long sections (corresponding to a 0.2 Rf range), which represent a particular lipid class and put into the HS vials. A 0.5 M

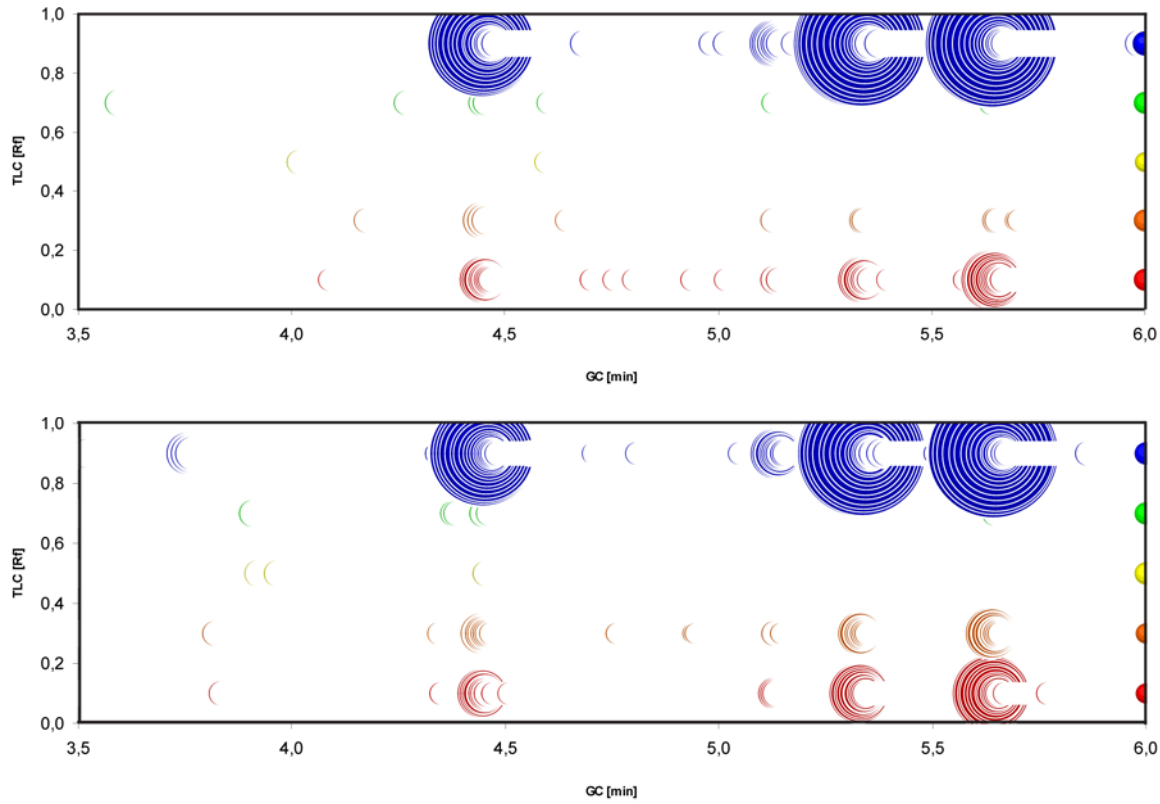

Figure 5. TLCxGC chromatograms of two different pumpkin seed oils. The X axis shows the retention times (Rt) of substances separated using GC, the Y axis represents spot positions on TLC lanes (as Rf values), and the circle size describes the signal intensity.

KOH methanol solution was dropped onto the silica gel side. The HS vials were left at room temperature in order to allow the transesterification reaction to occur. Afterwards, the sampling was carried out under the described headspace conditions. Samples were analyzed using a GC system equipped with a flame ionization detector (FID). Examples of two pumpkin seed oil TLCxGC comprehensive chromatograms are shown in Figure 5.

3. Conclusion

Analytical techniques based on the coupling of several separation and detection systems, such as ion chromatography-mass spectrometry, thin-layer chromatography-mass spectrometry and thin-layer chromatography-gas chromatography, are valuable methods for the quantitative determination of selected components in biological samples. The presented analytical tools have remarkable capabilities to resolve complex carbohydrate and lipid mixtures and to quantify targeted analytes with high sensitivity. Furthermore, ESI ionization and ion trap mass spectrometry having MS^n capabilities can provide complete structural elucidation with stereochemistry information. Thus, the combination of electrospray with quadrupole ion trap mass spectrometers is promising techniques for future research in carbohydrate metabolism. Ion traps combine advantages of quadrupole filters, e.g., low costs, ease of operation, unit-mass resolution and not too severe vacuum restrictions. Furthermore, ion traps provide better sensitivity compared to conventional quadrupole filters. Regarding the preliminary studies in IC-MS field it can be predicted that additional sensitive applications can be expected in this area of research in the near future.

The proof that the potential in the field of carbohydrate analysis by IC-PAD is still growing, is the newly developed two-pulse waveform. Thus, the novel view of the electrochemistry of gold is indicated. The introduced two-step waveform with higher sampling frequency enables the PAD detection to become ideally suitable for emerging capillary IC systems.

4. References

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Povzetek

V prispevku je podan pregled razvoja nekaterih analiznih metod za kvantitativno določanje spojin na prehrambenem področju, s poudarkom na izbranih sklopljenih analiznih tehnikah. Večji del je posvečen pregledu razvoja analiznih orodij za rutinske analize ogljikovih hidratov in maščob v bioloških vzorcih, mnoge med njimi vpeljane v našem laboratoriju. Rokovanje z biološkimi vzorci predstavlja velik izziv, saj lahko endogene spojine zakrijejo tarčni analit in kjer koelucijski učinki večih spojin, prisotnih v različnih količinah, lahko ovirajo integracijo vrhov izbranih analitov. Da premostimo omenjene izzive, so bile v laboratorijsko prakso uvedene sklopljene analizne tehnike. Predstavljene so nekatere rešitve, s posebnim poudarkom na učinkovitem vmesniku med tenkoplastno kromatografijo in masno spektroskopijo ter povezava med tenkoplastno kromatografijo in plinsko kromatografijo. Za premagovanje bioanalitskih problemov je bila pred kratkim na Kemijskem inštitutu v Ljubljani inštalirana sklopitev ionskega kromatografa in masnega spektrometra, hibrid RF/DC kvadrupola in linearne ionske pasti. Predstavljene so razvite metode za določevanje ogljikovih hidratov, uporabljajoč ionsko kromatografijo v povezavi z masno spektrometrijo in ionsko kromatografijo s pulzno amperometrično detekcijo. Obstoječ pregledni članek predstavlja naše raziskovalno delo na področju modernih sklopljenih tehnik. V članku so izpostavljeni signifikantni doprinosi na področju analitike ogljikovih hidratov in lipidov z izbranimi sklopljenimi tehnikami.