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ABSTRACT OF THE DOCTORAL THESIS

DEVELOPMENT AND APPLICATION OF NEW MASS SPECTROMETRIC METHODS IN CLINICAL CHEMISTRY

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I. INTRODUCTION, AIM

The importance of early diagnosis of illnesses and their prompt treatment have been long recognized in human health care. Modern analytical methodologies continue to play an important role in the field of clinical diagnostics; the public interest is focusing on the introduction of new, fast and selective methods to improve clinical practice. In the last decade mass spectrometry revolutionized biochemical and clinical research. The newly developed ionization techniques and mass analyzers opened a new horizon for the investigation non-volatile and high molecular mass compounds such as metabolites, proteins, glycoproteins. Their analysis became possible even in complex biological matrices, so investigation of body fluids (such as blood, urine) became feasible. Most of these compounds can be analyzed using the robust, selective and fast HPLC-MS and MS/MS techniques without preliminary chemical derivatization, thus these methods are often replacing gas chromatography - mass spectrometry (GC-MS).

Applying sensitive, high throughput screening techniques several illnesses can be screened simultaneously using minimal amounts of sample (e.g. a few microliters of blood) and even the whole population of a country can be screened at a reasonable price (as it is done in the United States, United Kingdom or Germany today).

Despite the large number of publications describing the importance of metabolic screening performed by mass spectrometry, its potential in clinical science is still not fully exploited. There are ongoing developments to increase the speed of analysis, the number of the detectable compounds and the number of detectable illnesses.

The aim of the present doctoral thesis was

a) to develop and to apply new mass spectrometry based methods that can be used in clinical research and,

b) to introduce mass spectrometry based diagnostic methods into the Hungarian clinical practice.

II. EXPERIMENTAL

Amino acid and acylcarnitine analyses of dried blood spots were performed by using positive electrospray ionization followed by tandem mass spectrometric detection on a Perkin Elmer API 2000 Triple Quadrupole mass spectrometer.

Analysis of fatty acids, triacylglycerols and other apolar compounds were performed applying both positive and negative atmospheric pressure chemical ionization mass spectrometry on the same Perkin Elmer API 2000 Triple Quadrupole mass spectrometer coupled to two Perkin Elmer Series 200 micropumps. Chromatography was performed on a C18 ec Purospher Star column (3 μm particle size, 55 mm x 2 mm i.d.).

Human alpha-1-acid glycoprotein analyses were performed on a 9.4 Tesla Bruker APEX Fourier Transform Ion Cyclotron Resonance mass spectrometer equipped with an electrospray ion source. Further instrumental and experimental parameters are given in my PhD thesis and in the original publications.

III. NEW RESULTS AND CONCLUSIONS

III.1. We introduced the modern, increasingly used electrospray tandem mass spectrometric screening method for detection of inherited metabolic disorders to Hungary. We have developed co-operations with several hospitals over the country. In the framework of this co-operation, we identified several metabolic disorders including amino acidurias and beta oxidation disorders. Now this technique is becoming to be a routinely used in Hungarian clinical practice. We developed an evaluation algorithm, which makes possible the simultaneous overview of large amount of patient-metabolite data pairs (see Figure 1).

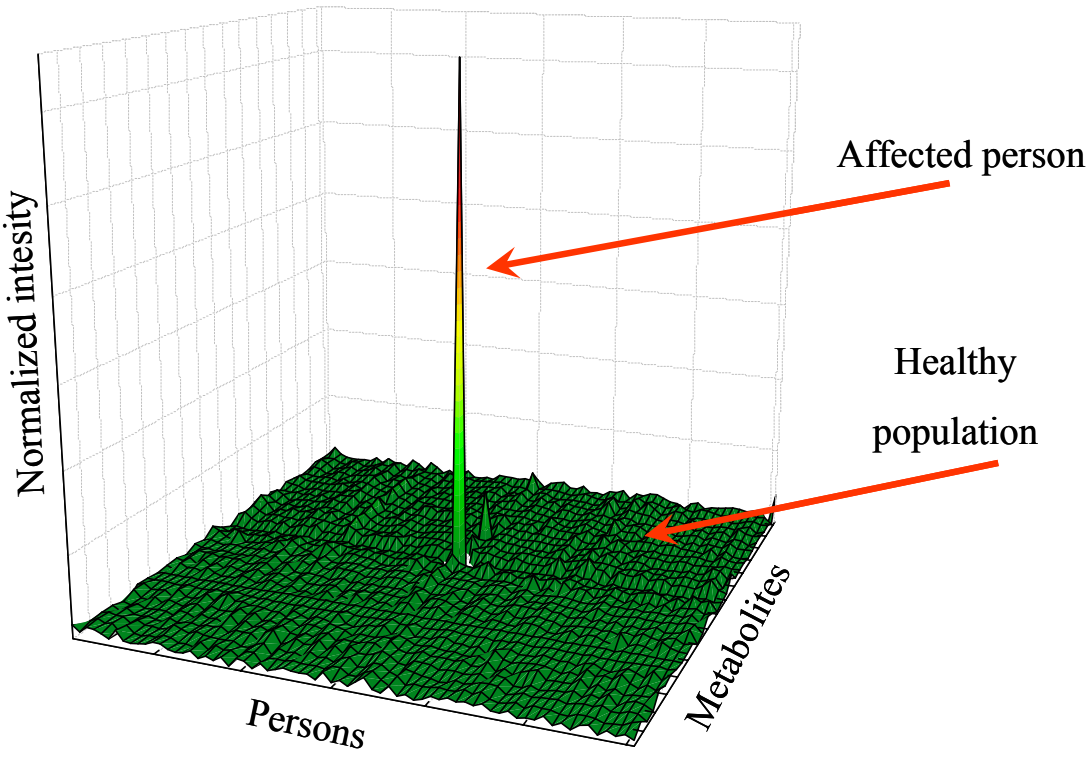

Figure 1. An affected child is separated as a sharp peak from the healthy population on the base of mass spectrometric data.

III.2. We developed a tandem mass spectrometric method for the determination of amino acids in dried blood spots that requires neither the use of chemical derivatization nor chromatographic separation. The method is based on solid phase extraction followed by direct injection and positive electrospray tandem mass spectrometric analysis in multiple reaction monitoring (MRM) mode. Solid phase extraction of amino acids was performed using anion exchange cartridges pre-equilibrated with acetate counter ions. Since chemical derivatization is not required, several drawbacks that of the classical butylation method are eliminated, such as conversion of glutamine to glutamic acid, asparagine to aspartic acid, hydrolysis of acylcarnitines etc. Calibration curves, detection limits, interday- and intraday uncertainties suggest that this method is a good alternative to the classical butylation method. Furthermore, this method is sufficiently flexibile to incorporate analysis of new compounds for screening purposes. In a critical case Citrullinaemia (not screened disorder in Hungary) was identified using the developed technique.

III.3. Our studies have shown that the endogenous sodium content of blood exhibits significant suppression of the amino acids, leading to more than 90 % loss of signal intensities.

III.4. The developed method was successfully applied for the distinction among leucine, isoleucine and hydroxyproline isobars without the need of preliminary chromatographic separation. During analysis, molecular ions produced by electrospray ionization are fragmented using collisional induced dissociation (CID) at 40-50 eV collisional energy. Under such conditions amino acids yield fragments that can be used for unequivocal identification.

III.5. We developed an HPLC-MS method for the investigation of fatty acids and other apolar compounds. The method is based on reversed phase separation using partly miscible water-methanol-n-hexane solvents and provides extreme short analysis times (see Figure 2). The separation mechanism is believed to occur in the pores at the beginning of the column and not along the column, thus it is significantly different from the traditional approaches. The combination of fast analysis time (Figure 2) and low solvent consumption is of particular importance in high throughput studies. Detection was performed using atmospheric pressure chemical ionization, detection limits were in the low picogram range for fatty acids.

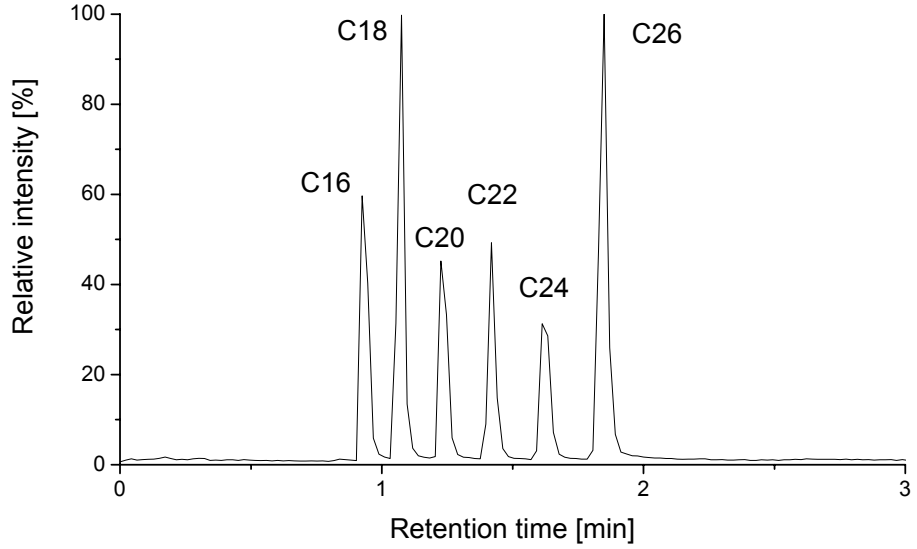

Figure 2. HPLC-MS total ion chromatogram of a fatty acid mixture.

III.6. The developed method is suitable to analyze fatty acids without the need of preliminary chemical derivatization and was successfully applied for the determination of very long chain fatty acid (C18-C26) content of blood. Validation data and short analysis times suggest that this method may be used as an alternative to the very time-consuming GC-MS (gas

chromatography mass spectrometry) methods used for screening peroxisomal disorders. The method is also suitable for the analysis of other apolar compounds with minimal method modification, as it was demonstrated in the case of triacylglycerols and sterols.

III.7. We have developed an FT-ICR (Fourier Transform Ion Cyclotron Resonance Mass Spectrometry) technique for the investigation of extremely heterogeneous protein-, glycoprotein mixtures, containing of several hundreds structurally closely related components. Using human alpha-1-acid glycoprotein (AGP) as model compound, we proved that intact (not digested) glycoproteins can be analyzed by electrospray ionization without preliminary chromatographic separation using large amount (50 %) of trifluoroethanol co-solvent.

III.8. An FT-ICR method including isolation and axialization of ions was developed and successfully applied for the monoisotopic resolution of certain variants of human AGP (Figure 3). Following calibration, atomic compositions corresponding to certain AGP variants could be determined. Using our results and observations found in the literature, it was possible to associate the experimentally obtained atomic compositions with glycoprotein structures. The results suggest that ultrahigh resolution mass spectrometry may play a significant role for investigating post-translational modifications in heterogeneous glycoprotein mixtures.

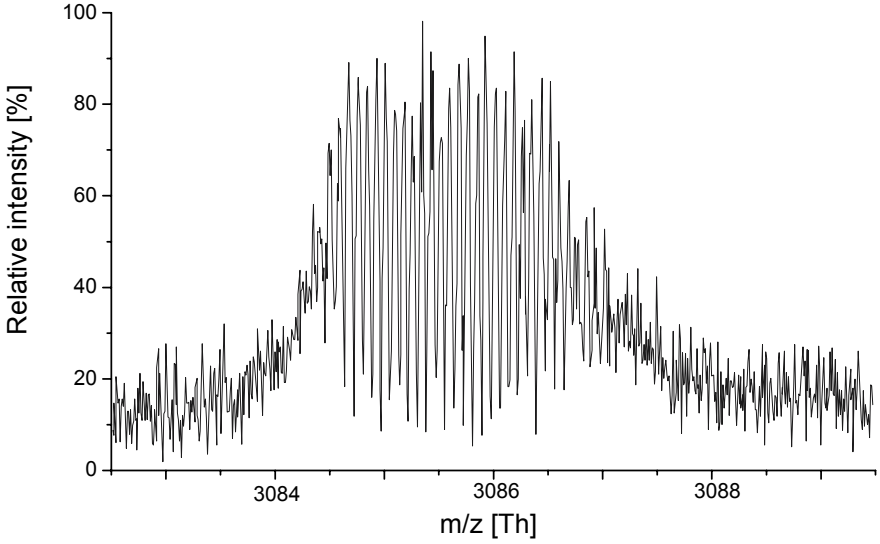

Figure 3. Ultrahigh resolution mass spectrum of the intact human AGP following electrospray ionization.

IV. USEFULNESS OF THE RESULTS

In the course of our work, we developed a new field of research in our laboratory and introduced tandem mass spectrometry based screening techniques to the Hungarian clinical community. We joined international efforts to investigate small and large molecular-weight compounds of biological and clinical importance (metabolomics, proteomics). Our method developments serve to improve currently used analytical tools in clinical diagnosis.

V. PUBLICATIONS

Accepted articles (related to the thesis):

1 Kornél Nagy, Zoltán Takáts, Ferenc Pollreisz, Teréz Szabó and Károly Vékey; Direct tandem mass spectrometric analysis of amino acids in dried blood spots without chemical derivatization for neonatal screening; ***Rapid Communications in Mass Spectromety*** **2003**; 17:983-990.

2 Kornél Nagy, Annamária Jakab, Jenő Feket, Károly Vékey; An HPLC-MS approach for analysis of very long chain fatty acids and other apolar compounds on octadecyl-silica phase using partly miscible solvents.; ***Analytical Chemistry*** **2004;** 76:1935-1941.

3 Kornél Nagy, Károly Vékey, Tímea Imre, Krisztina Ludányi, Mark P. Barrow, Peter J. Derrick; Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry of Human Alpha-1-acid Glycoprotein; ***Analytical Chemistry*** **2004;** 76:4998-5005.

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Further papers (directly not related to the thesis):

6 Kornél Nagy, Edina Epacher, Peter Staniek, Béla Pukánszky; Hydrolytic stability of phenolic antioxidants and its effect on their performance in high-density polyethylene; ***Polymer degradation and stability*** **2003**; 82:211-219.

7 Annamária Jakab, Kornél Nagy, Károly Héberger, Károly Vékey and Eszter Forgács; Differentiation of vegetable oils by mass spectrometry combined with statistical analysis; ***Rapid Communications in Mass Spectrometry*** **2002**; 16:2291-2297.

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1 Kornél Nagy, Károly Vékey; Tandem Mass Spectrometry in Clinical Chemistry: the Metabolic Profiling Challenge; *Applied Biosystems Seminar*; 20-21 May, **2003**; Prague, Czech Republic

Oral presentations (related to the thesis):

2 Kornél Nagy, Zoltán Takáts, Károly Vékey, Novel Aspects of Inherited Metabolic Disorder Screening by Tandem Mass Spectrometry, ***19th Informal Meeting on Mass Spectrometry***; 29 Apr-3 May, **2001;** Noszvaj, Hungary

3 Kornél Nagy, Zoltán Takáts, Károly Vékey, Tandem Mass Spectrometry in Clinical Diagnostics, ***NATO Advanced Studies Institute,*** *New Frontiers in Mass Spectrometry. Recent Developments in Instrumentation and Applications*; 09.01-11. **2001**; Tetouan, Marocco

4 Nagy Kornél, Takáts Zoltán, Vékey Károly, Új Módszerek az Újszülöttek Anyagcsere-rendelleneségének Tandem Tömegspektrometriás Szűrésében; IV. ***Doktori Kémiai Iskola***; Május 20-22. **2001**; Mátraháza

5 Kornél Nagy, Károly Vékey, Peter Derrick, ES-FT-ICR investigation of intact highly sialylated glycoproteins using human alpha-1-acid glycoprotein as model compound.; ***21st Informal Meeting on Mass Spectrometry***; 11-15 May, **2003;** Antwerp

6 Nagy Kornél, Vékey Károly; Tandem tömegspektrometria az orvosi kémiában, ***Kutatóközponti Tudományos Napok***; május 28-29. **2003**; MTA KKKI, Budapest

7 Kornél Nagy, Mária Bihari, Károly Vékey; New approach in Clinical Chemistry: application of mass spectrometry, ***Advisory Board Meeting***; June 17-19. **2003;** HAS CRC, Budapest

8 Kornél Nagy; Application of mass spectrometry in clinical chemistry; ***RIGI Meeting 2003***; 30-31. Oct. **2003;** Dorint Hotel Blüemlisalp, Beatenberg, Switzerland

Posters (related to the thesis):

1 Kornél Nagy, Zoltán Takáts, Ferenc Pollreisz, Károly Vékey, Analysis of long and very long chain fatty acids from human plasma by atmospheric pressure chemical ionization tandem mass spectrometry; ***20th Informal Meeting on Mass Spectrometry***; 12-16 May, **2002;** Fiera di Primiero, Italy - **ISBN 88 86281 69 3**

2 Kornél Nagy, Zoltán Takáts, Károly Vékey; Electrospray tandem mass spectrometric analysis of 24 amino acids from human blood spot without chemical derivatization used for neonatal screening; ***50th Annual Conference on Mass Spectrometry and Allied Topics, American Society for Mass Spectrometry***; 2-6 jun. **2002;** Orlando, FL, USA

3 Kornél Nagy, Jenő Fekete, Károly Vékey; A novel HPLC-MS method for the analysis of fatty acids and other apolar compounds on octadecyl-silica phase; ***Advances in Chromatography and Electrophoresis - Conferentia Chemometrica 2003 (ACE&CC 2003)***; 27-29. Oct. **2003;** Budapest, Agro Hotel- ***ISBN 963-508-391-2***

4 Kornél Nagy, David Bongiorno, Pasquale Agozzino, Giuseppe Avellone, Ceraulo Leopoldo, Károly Héberger, Károly Vékey; A simple High Performance Liquid Chromatography - Mass Spectrometry approach combined with Linear Discriminant Analysis for the characterization of top quality olive oils.; ***22nd Informal Meeting on Mass Spectrometry***; May 2-6, **2004;** Tokaj, Hungary

5 Annamária Jakab, Kornél Nagy, Károly Vékey, Esther Forgács; Analysis of plant oil triacylglycerols by HPLC-APCI-MS and MALDI-

TOF-MS; ***20th Informal Meeting on Mass Spectrometry***; 12-16 May, **2002;** Fiera di Primiero, Italy - ***ISBN 88 86281 693***

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7 Martin Zeller, Mark Barrow, Kornel Nagy, Simone Koenig and Peter J. Derrick; Electron capture dissociation (ECD): A tool for analysis of phosphorylated proteins with Fourier transform ion cyclotron resonance (FTICR) mass spectrometry; *Intermolecular Associations in 2D and 3D* - ***Biochemical Society Focused Meeting Joint Meeting with The British Biophysical Society***; 19 - 20 June **2003;** University of Nottingham, UK

8 Martin Zeller, Mark Barrow, Kornel Nagy, Simone Koenig and Peter J. Derrick; Analysis of phosphorylated proteins with Fourier transform ion cyclotron resonance (FTICR) mass spectrometry. Electron capture dissociation (ECD) and sustained off-resonance irradiation (SORI) collision-induced dissociation (CAD); ***16th IMSC***; Aug 31- Sep 5, **2003;** Edinburgh, Scotland, UK

9 Jenő Fekete, Kornél Nagy, Károly Vékey; Optimalization of fast gradient elution for HPCL-MS/MS; ***5th Balaton Symposium on High-Performance Separation Methods***; September 3-5, **2003;** Siófok, Hungary - ***ISBN 963-508-391-2***

10 Annamária Jakab, Károly Héberger, Kornél Nagy, Károly Vékey, Eszter Forgács; Characterization of plant oils based on their triacylglicerol

content by HPLC/APCI-MS and MALDI-TOFMS combined with linear discriminant analysis; ***Advances in Chromatography and Electrophoresis - Conferentia Chemometrica 2003 (ACE&CC 2003);*** 27-29. Oct. **2003**; Budapest, Agro Hotel - ***ISBN 963-7067-108***

Further posters (directly not related to the thesis):

11 Kornél Nagy, Edina Epacher, Jan Malik, Peter Staniek, Béla Pukánszky; Hydrolytic stability of phenolic antioxidants; effect on their performance under extractive conditions; ***Second International Conference on Polymer Modification, Degradation and Stabilization*****;** June 30 - July 04, **2002;** Budapest, Hungary **-** ***ISBN 963 420 723 5***