

**CECE 2017**  
**14<sup>th</sup> International Interdisciplinary Meeting on Bioanalysis**  
**Veszprém, Hungary, 8-11 October, 2017**

**ISBN: 978-963-396-103-2**

**Proceedings editors:** András Guttman, Herbert Lindner, Gábor Járvás,  
László Hajba, Brigitta Mészáros

MTA-PE Translational Glycomics Research Group, Research Institute for  
Biomolecular and Chemical Engineering, University of Pannonia  
Egyetem u. 10, H-8200 Veszprém, Hungary

**Organized by:**

MTA-PE Translational Glycomics Research Group, Research Institute for  
Biomolecular and Chemical Engineering, University of Pannonia  
Egyetem u. 10, H-8200 Veszprém, Hungary

**Organizing committee:** András Guttman, Herbert Lindner, Gábor Járvás,  
László Hajba, Brigitta Mészáros, Márton Szigeti, Beáta Borza, Balázs Reider

**Webmaster:** Brigitta Mészáros, Márton Szigeti, Gábor Járvás,  
László Hajba

**Find the meeting history and more at [cece.mik.uni-pannon.hu](http://cece.mik.uni-pannon.hu)**

## **Foreword**

Welcome to CECE 2017, the 14<sup>th</sup> International Interdisciplinary Meeting on Bioanalysis. As in the previous years, it is our goal to: “bring together scientists from different disciplines who may not meet at other meetings”. CECE Junior will follow two days of invited speaker’s lectures and poster sessions will be open during all three days. The organizers want to thank all invited speakers, sponsors and participants for their continuing support. Please, check our web at [cece.mik.uni-pannon.hu](http://cece.mik.uni-pannon.hu) for more information about the history, programs, photos and videos from the previous years.

András Guttman and Herbert Lindner

Veszprém, October 6, 2017



# Conference program of CECE 2017

University of Pannonia, Veszprem, Hungary, October 08-11, 2017

## Day 1 – Monday, 9 October, 2017 /Conference Center, Building B/

08:00 – 15:00      **Registrations**

09:00 – 09:15      **CECE 2017 – Opening remarks:  
Andras Guttman and Herbert Lindner**

09:15 – 09:45      **Bianca Avramovitch**

*Senior Director, Global Analytical R&D, Teva, Kfar Saba, Israel*

EMERGING ANALYTICAL TECHNOLOGIES AND NOWADAYS  
PHARMACEUTICAL DRUG PRODUCTS DEVELOPMENT  
CHALLENGES

09:45 – 10:15      **Jeff Chapman**

*Senior Director CE Business Unit SCIEX, Brea, California, USA*

ANALYSIS OF PROTEIN GLYCOLYSATION OF HIGH  
BIOMEDICAL IMPORTANCE BY CAPILLARY  
ELECTROPHORESIS AND ITS COMBINATION WITH MASS  
SPECTROMETRY

10:15 – 10:45      **Coffee break**

10:45 – 11:15      **Danilo Corradini**

*Research Director, Italian National Research Council, Rome, Italy*

EFFECTS OF METAL IONS, ALKYLAMINES AND ORGANIC  
SOLVENTS ON THE SEPARATION OF BIOMOLECULES BY  
CAPILLARY ELECTROPHORESIS

- 11:15 – 11:45      **Mercedes de Frutos**  
*Research Scientist, Department of Instrumental Analysis in the Institute of Organic Chemistry (IQOG) of Spanish Council Research (CSIC), Madrid, Spain*  
ANALYTICAL METHODOLOGIES IN THE SEARCH OF GLYCOBIOMARKERS
- 11:45 – 12:15      **Károly Vékey**  
*Professor, Hungarian Academy of Sciences, Research Centre for Natural Sciences, Budapest, Hungary*  
PROTEIN GLYCOSYLATION AND MASS SPECTROMETRY
- 12:15 – 14:15      **Lunch break and Poster session /B-C corridor/**
- 14:15 – 14:45      **Frantisek Foret**  
*Deputy Head, Institute of Analytical Chemistry of the Czech Academy of Sciences, Brno, Czech Republic*  
TO INTERFACE OR NOT TO INTERFACE IN CE-MS. ONE STORY, TWO POINTS OF VIEW
- 14:45 – 15:15      **András Gelencsér**  
*Rector, University of Pannonia, Veszprém, Hungary*  
BRIDGING SCALES AND DISCIPLINES: SOOT NANOPARTICLES IN THE ATMOSPHERE
- 15:15 – 15:45      **Norberto A. Guzman**  
*Department of Immunoengineering-Bioanalysis Princeton Biochemicals, Inc. Princeton, New Jersey, USA*  
ADVANCEMENTS IN MINIATURIZED IMMUNOAFFINITY CAPILLARY ELECTROPHORESIS: SHIFTING PARADIGMS IN BIOANALYSIS TO MONITOR WELLNESS, DISEASE, AND TREATMENT EFFECTIVENESS

16:30 – 18:00      **City tour for the invited speakers hosted by Andras Guttman**

18:00 – 19:00      **Wine tasting /B-C corridor/**

**Day 2 – Tuesday, 10 October, 2017 /Conference Center, Building B/**

09:30 – 10:00      **Svetlana M. Krylova**

*Senior Research Associate, Centre for Research on Biomolecular Interactions, York University, Toronto, Canada*

CAPILLARY ELECTROPHORESIS-BASED ANALYSIS OF ENZYMATIC MODIFICATIONS OF PROTEINS AND DNA

10:00 – 10:30      **Sergey N. Krylov**

*Director, Centre for Research on Biomolecular Interactions, York University, Toronto, Canada*

PROSPECTIVES OF CAPILLARY ELECTROPHORESIS IN VALIDATION OF MOLECULAR BIOMARKERS OF CANCER

10:30 – 11:00      **Coffee break**

11:00 – 11:30      **Katalin Medzihradzsky**

*Adjunct Professor, University of California, San Francisco, USA*

NEGLECTED POST-TRANSLATIONAL MODIFICATIONS: EXTRACELLULAR O-GLYCOSYLATION AND PHOSPHORYLATION

11:30 – 12:00      **Marek Minarik**

*Director, Centre for applied genomics of solid tumors (CEGES), Genomac Research Institute Prague, Czech Republic*

CANCER DIAGNOSIS AND MONITORING BY CE-BASED LIQUID BIOPSY ASSAY

- 12:00 – 14:00      **Lunch break and Poster session /B-C corridor/**
- 14:00 – 14:30      **Herbert Lindner**  
*Innsbruck Medical University, Biocenter, Division of Clinical Biochemistry  
Innrain 80, A-6020 Innsbruck, Austria*  
ADVANCES IN THE SEPARATION OF CO- AND POST-TRANSLATIONAL PROTEIN MODIFICATIONS USING CE-MS
- 14:30 – 15:00      **Pier Giorgio Righetti**  
*Professor, Politecnico of Milano, Milano, Italy*  
A NOVEL CLASS OF MINIATURIZED SENSORS FOR BIO-ANALYSIS
- 15:00 – 15:30      **Maria Fernanda Silva**  
*Principal Investigator, National University of Cuyo, Mendoza, Argentina*  
NOVEL APPROACHES MEDIATED BY TAILOR-MADE GREEN SOLVENTS FOR THE CHARACTERIZATION OF BIOLOGICALLY ACTIVE COMPOUNDS
- 15:30 – 16:00      **Coffee break**
- 16:00 – 16:30      **Robert Weinberger**  
President at CE Technologies, Inc, New York, USA  
FOUR DECADES OF CAPILLARY ELECTROPHORESIS
- 16:30 – 17:00      **John R. Yates**  
*Professor, Department of Chemical Physiology and Molecular and Cellular Neurobiology, The Scripps Research Institute, La Jolla, California, USA*  
Csaba Horváth Lectureship 2017 Presentation



17:15 – 18:30            **Opening of Ervin sz. Kováts exhibition /Building I, 8th floor/**

19:00 – 21:00            **Conference dinner /B-C corridor/**

21:00 – Wednesday morning            **Veszprém's night life welcome you!**

### **Day 3 – Wednesday, 11 October, 2017 /Conference Center, Building B/**

09:00                      **Opening of Plenary Lecture**

09:00 – 09:30            **Ferenc Kilár**

*Professor, University of Pécs, Pécs, Hungary*

HOW TO DEVELOP CAPILLARY ELECTROPHORESIS?

09:30                      **Opening of CECE Junior Session**

09:30 – 09:45            **Hana Nevídalová**

*Department of Biochemistry, Masaryk University, Brno, Czech Republic*

COMPARISON OF FOUR METHODS USED FOR PLASMA PROTEIN-DRUG INTERACTION STUDIES

09:45 – 10:00            **Cynthia Nagy**

*University of Debrecen, Department of Inorganic and Analytical Chemistry*

CHARACTERIZATION OF A MICROFLUIDIC ENZYME REACTOR FOR THE RAPID PROTEOLYSIS OF HUMAN TEAR SAMPLES

- 10:00 – 10:15      **Vojtěch Ledvina**  
*Faculty of Science, Masaryk University, Brno*  
MINIATURIZED DEVICE FOR SINGLE-CELL ANALYSIS OF  
CASPASE-3 AND 7 ACTIVATION USING BIOLUMINESCENCE  
CHEMISTRY
- 10:15 – 10:30      **Brigitta Mészáros**  
*Horváth Csaba Memorial Institute of Bioanalytical Research,  
University of Debrecen, Debrecen, Hungary*  
DEVELOPMENT OF A HIGH-RESOLUTION SDS SEPARATION  
GEL FOR PROTEIN ANALYSIS BY CGE-MS
- 10:30 – 11:00      **Coffee break**
- 11:00 – 11:15      **Mate Szarka**  
*Horváth Csaba Memorial Institute of Bioanalytical Research,  
University of Debrecen, Debrecen, Hungary*  
IN FLIGHT ANALYSIS OF ASTRONAUT'S SERUM  
IMMUNOGLOBULINS DURING DEEP SPACE MISSIONS; THE  
NEXT LEAP IN CAPILLARY ELECTROPHORESIS
- 11:15 – 11:30      **Jasna Hradski**  
*Department of Analytical Chemistry, Faculty of Natural Sciences,  
Comenius University in Bratislava, Slovakia*  
MICROCHIP ELECTROPHORESIS WITH CONDUCTIVITY  
DETECTION IN THE ANALYSIS OF PHARMACEUTICAL AND  
BIOLOGICAL SAMPLES
- 11:30 – 11:45      **Anikó Kilár**  
*Institute of Bioanalysis, University of Pécs, Pécs, Hungary*  
PHOSPHOGLYCOLIPID PROFILING OF BACTERIAL  
ENDOTOXINS

- 11:45 – 12:00      **Kateřina Hanáková**  
*National Centre for Biomolecular Research, Faculty of Science,  
Masaryk University, Brno, Czech Republic*  
SEMIQUANTITATIVE ASSESSMENT OF DISHEVELLED 3  
PHOSPHORYLATION STATUS BY MASS SPECTROMETRY
- 12:00 – 13:30      **Lunch break and Poster session /B-C corridor/  
Poster removal after lunch.**
- 13:30 – 13:45      **Tomas Vaclavek**  
*Department of Biochemistry, Faculty of Science, Masaryk University,  
Brno, Czech Republic*  
MICROFLUIDIC CELL COUNTING DEVICE BASED ON  
RESISTIVE PULSE SENSING
- 13:45 – 14:00      **Boglárka Dönczö**  
*Horváth Csaba Memorial Institute of Bioanalytical Research,  
University of Debrecen, Debrecen, Hungary*  
MOLECULAR GLYCOHISTOPATHOLOGY BY CAPILLARY  
ELECTROPHORESIS
- 14:00 – 14:15      **Andrea Celá**  
*Department of Biochemistry, Faculty of Science, Masaryk University,  
Brno, Czech Republic*  
ON-CAPILLARY DERIVATIZATIONS OF AMINO ACIDS IN  
CAPILLARY ELECTROPHORESIS USING NAPHTHALENE-2,3-  
DICARBOXALDEHYDE/SODIUM CYANIDE
- 14:15 – 14:30      **Beáta Borza**  
*Horváth Csaba Memorial Institute of Bioanalytical Research,  
University of Debrecen, Debrecen, Hungary*  
COMPARATIVE N-GLYCOSYLATION ANALYSIS OF A  
BIOSIMILAR AND ITS INNOVATOR PRODUCT BY CE-LIF

14:30 – 14:45

**Balázs Reider**

*MTA-PE Translational Glycomics Research Group, University of Pannonia, Veszprém, Hungary*

PROSTATE-SPECIFIC ANTIGEN (PSA) IMMOBILIZATION  
FROM BIOLOGICAL SAMPLES FOR CAPILLARY  
ELECTROPHORESIS (CE) ANALYSIS

14:45 – 14:50

**CECE 2017 – Closing remarks:**

**Andras Guttman and Herbert Lindne**

## List of poster presentations

- P1. IDENTIFICATION OF O-GLYCOSYLATED (N-TERMINAL) PRO-B-TYPE NATRIURETIC PEPTIDE FORMS IN BLOOD PLASMA OF PATIENTS WITH SEVERE HEART FAILURE  
Benno Amplatz, Bernhard Halfinger, Angelika Hammerer-Lercher, Bettina Sarg, Leopold Kremser, and Herbert H. Lindner
- P2. CAPILLARY ISOTACHOPHORESIS DETERMINATION OF TRACE OXIDIZED GLUTATHIONE IN BLOOD  
Róbert Bodor, Andrea Nečasová, Alena Pechová, and Marián Masár
- P3. GLYCOSIMILARITY INDEX FOR BIOTHERAPEUTICS  
Andras Guttman, Beata Borza, Marton Szigeti, Akos Szekrenyes, Laszlo Hajba
- P4. SEMIAUTOMATION OF LIQUID HANDLING FOR MICROELECTROMEMBRANE EXTRACTION OF COMPLEX SAMPLES  
Miloš Dvořák, Andrea Šlampová, And Pavel Kubáň
- P5. DEVELOPMENT OF GRADIENT RP-HPLC METHOD FOR CHARACTERIZATION OF AMINO ACID COMPOSITION OF DEGRADATION PRODUCTS OF HUMIC ACIDS ISOLATED FROM PEAT AND SOIL AFTER PRE-COLUMN DERIVATIZATION  
Róbert Góra, Milan Hutta, Erik Beňo, and Natália Bielčíková
- P6. STUDY OF VARIOUS PARAMETERS INFLUENCING CONTENT OF EXHALED BREATH CONDENSATE WITH IMPORTANCE FOR DIAGNOSIS OF GASTROESOPHAGEAL REFLUX DISEASE  
Michal Greguš, Pavol Ďurč, Júlia Lačná, František Foret And Petr Kubáň
- P7. DETERMINATION OF THE OLIGOSACCHARIDE COMPOSITION IN WORT SAMPLES BY CAPILLARY ELECTROPHORESIS  
Tamás Gábor Szilágyi, Beáta Hegyesné Vecseri, Zsuzsanna Kiss, László Hajba, András Guttman
- P8. MULTISCALE MODELING OF RECTIFYING BIPOLAR NANOPORE  
Z. Ható, M. Valiskó, T. Kristóf, D. Gillespie, D. Boda
- P9. CFD MODELLING BASED INVESTIGATION OF THE ELECTROOSMOTIC AND FORCED CONVECTION FLOW IN CAPILLARY ELECTROPHORESIS APPLICATIONS  
Gábor Járvas, Márton Szigeti, and András Guttman
- P10. SEARCH FOR MULTIPLE MYELOMA GLYCOBIOMARKERS BY CE-LIF  
Zsuzsanna Kovács, Anna Farkas, and András Guttman

- P11. BIOBANKING OF PATIENT SAMPLES FOR GENO – GLYCOMIC LUNG DISEASE BIOMARKER STUDIES  
Renáta Kun, Eszter Csánky, Miklós Szabó, Zsolt Rónai, Zsuzsanna Kovács, Márton Szigeti, Gábor Járvas, László Hajba, Boglárka Dönczö, András Guttman
- P12. SINGLE-CELL ANALYSIS OF ACTIVE CASPASE 3/7 IN APOPTOTIC CELLS  
Vojtěch Ledvina and Karel Klepárník
- P13. APPLICATION OF AMINO ACIDS AND PEPTIDES FOR OLIGOSACCHARIDE LABELING  
Jana Krenkova, Jan Partyka, and Frantisek Foret
- P14. CAPILLARY ELECTROPHORETIC ANALYSIS OF EXHALED BREATH CONDENSATE IN THE DIAGNOSIS OF GASTROESOPHAGEAL REFLUX DISEASE  
Petr Kubáň, Pavol Ďurč, Júlia Lačná, Michal Greguš, František Foret, Jiří Dolina, Štefan Konečný, Martina Doubková, Dagmar Kindlová, Eva Pokojová, and Jana Skříčková
- P15. USING NANOPORES AS SENSORS: A COMPUTER SIMULATION STUDY  
Eszter Mádai, Mónika Valiskó, and Dezső Boda
- P16. QUANTITATIVE ASSESSMENT AND AUTOMATIC ID OF GLYCANS OF CQA IMPORTANCE IN BIOLOGICS DEVELOPMENT  
Andras Guttman, Marton Szigeti, Brigitta Meszaros, Jeff Chapman
- P17. DEVELOPMENT OF AN UHPLC-MS/MS METHOD FOR ANALYSIS OF A NOVEL CARDIOPROTECTIVE AGENT JAS 2 AND ITS METABOLITE  
Hana Piskáčková, Petra Reimerová, Jaroslav Roh, Jana Sedláková, and Petra Štěrbová Kovaříková
- P18. *IN VITRO* BIOACTIVATION STUDY OF A NEW ANTIPROLIFERATIVE DRUG SOBUZOXANE IN BIOLOGICAL MATRICES  
Petra Reimerová, Anna Jirkovská, Hana Piskáčková, Galina Karabanovich, Jaroslav Roh, and Petra Štěrbová-Kovaříková
- P19. COMBINATION OF PREPARATIVE ISOELECTRIC FOCUSING AND MALDI-TOF MS FOR IDENTIFICATION OF COLORED BACTERIA  
Jiří Šalplachta, Marie Horká, Filip Růžička, and Karel Šlais
- P20. DETECTING MONO-PHOSPHORYLATED ISOBARIC PEPTIDES USING CESI-MS  
Bettina Sarg, Klaus Faserl, and Herbert H. Lindner
- P21. COLD PLASMA: THE WAY TO IMPROVE THE SALD ICP MS ANALYSIS REPEATIBILITY  
Marek Stiborek, Jan Preisler, Masoud Shekargoftar, Viktor Kanický, and Jakub Kelar
- P22. THE POWER OF GU AND THE IMPORTANCE OF TEMPERATURE CONTROL  
András Guttman, Márton Szigeti, Jeff Chapman

- P23. DETERMINATION OF HOMOCYSTEINE IN URINE AND SALIVA BY MICROCHIP ELECTROPHORESIS  
Peter Troška, Anna Mandžáková, Jasna Hradski, Juraj Ševčík, and Marián Masár
- P24. ANALYSIS AND IDENTIFICATION OF POLYPHENOLIC COMPOUNDS IN GREEN FOODS USING A COMBINATION OF HPLC-ESI-IT-TOF-MS/MS  
Pavel Škvára, Júlia Kezmanová, Csilla Mišlanová, Andrea Vojs Staňová
- P25. SILICA MONOLITHIC CAPILLARY COLUMNS FOR HILIC SEPARATIONS  
Dana Moravcová and Josef Planeta
- P26. MOLECULAR GLYCOHISTOPATHOLOGY BY CAPILLARY ELECTROPHORESIS  
Boglárka Dönczö and András Guttman
- P27. ENHANCED SEPARATION AND CHARACTERIZATION OF DEAMIDATED PEPTIDES WITH CE-MS  
Klaus Faser, Bettina Sarg, and Herbert H. Lindner

## About the invited speakers



**Bianca Avramovitch** is Senior Director in the R&D of Teva Pharmaceuticals, filling a number of positions: Global Analytical R&D Managers Forum Leader, Global AqBD Leader, Global ELN (Electronic Notebooks) Platform Business Owner, Head of Analytical Technologies Unit (ATU) of the Generic R&D IL.

In her global Teva R&D positions, Bianca provides leadership for highly skilled multi-disciplinary local and global teams, managing cutting edge technologies and computerized based research and development across sites and cultures.

Bianca holds a Ph. D. in Physical Organic Chemistry from the Hebrew University of Jerusalem, Israel. Following her post-doctoral studies at National Institutes of Health in US on histamine receptors, Bianca joined Teva and for the last 25 years she became an expert in a number of fields, developing a diversity of drug products: tablets, capsules, suspensions, sterile and injectable products, nasals, creams, and more. Analytical research and development areas which are up to today under her immediate responsibility are: solid state characterization aspects as polymorphism in Drug Substances and Products, particle size distribution by techniques such as laser diffraction and imaging, various physical properties characterization and measurement, Impurities mapping and characterization (identification by GC-MS and LC-MS/MS, by Capillary Electrophoresis).

Bianca is also holding the expert position in council of experts of the USP (United States Pharmacopoeia) being elected for a second cycle of 5 years. She is a member of the organizing committee of ISRANALYTICA. Bianca held various leadership functions in non-profit organizations and teaching environments, while lately she earned the "Making your family proud" value award of Teva for designing the Teva lab kit, the portable lab for parents to present the magic of chemistry in their children classrooms.



**Jeff Chapman** currently holds the position of Senior Director, CE Business, at SCIEX, a Danaher Company. Jeff and his team are responsible for the development, manufacturer, sales and support of electrodriven microscale separations technology at SCIEX. Jeff holds degrees in Biochemistry, Immunology and Neuroimmunology from the University of Saskatchewan and the University of Calgary . He joined Beckman Instruments in 1989 in Mississauga, where he was responsible for introducing Capillary Electrophoresis (CE) technology to Scientists across Canada. In 1995, he moved to Southern California, where he took on worldwide responsibility for CE. Beginning in 1998, Jeff moved into the development side of Beckman Coulter and has held the positions of Product Manager, Strategic Marketing Manager, Director of Scientific Alliances and Director of Global Marketing. Jeff transitioned the CE business over to SCIEX in 2013, integrating CE with nano-LC and micro-LC to form one of the largest microscale



separations operations in the World. Jeff has more than 30 years of experience with CE technology over his career, has served on myriad scientific committees and boards on this topic, and is cited often as an industry expert in this field. He is also serving currently as a member of the Strategic Planning Committee for MicroScale Bioseparations.



**Danilo Corradini** earned his Ph.D in Analytical Chemistry from the University of Rome “La Sapienza” in 1978, as a student of Michael Lederer, founder and first editor of the Journal of Chromatography. He investigates fundamental and practical aspects of capillary electrophoresis and HPLC in the field of phytochemistry, metabolomics and food chemistry at the Institute of Chemical Methodologies of the Italian National Research Council (CNR) in Montelibretti (Rome) Italy. Previously, he held academic appointments at Yale University (USA) in 1983-84, 1986 and 1996, working with the pioneer of HPLC Csaba Horváth. In recognition of his contribution to Separation science he has been honored with the Csaba Horváth Memorial Award from the Hungarian Separation Science Society in 2009, the Central European Group for Separation Science Award in 2011, and the Arnaldo Liberti Medal from the Italian Chemical Society in 2014.

He is in the editorial board of several international scientific journals and has been the Editor of the Second Edition of the Handbook of HPLC, published by CRC Press in 2010.



**Mercedes de Frutos** is Research Scientist of the Spanish Council Research (CSIC). She develops her work at the Department of Instrumental Analysis in the Institute of Organic Chemistry (IQOG) of CSIC. She carried out the studies for her Ph. D. in the field of chromatography at the IQOG-CSIC and defended her Ph. D. Thesis at the Autonoma University of Madrid (Spain). She specialized in immunochromatography for 2 years at Purdue University (USA). The work of her group focuses mainly on the development of HPLC and capillary electrophoresis methods and their coupling to immune-recognition techniques for analysis of proteins. She is mainly interested in the analysis of glycoproteins of relevance in the fields of doping control, biopharmaceuticals and disease biomarkers. Currently she focuses on prostate cancer markers.



**Frantisek Foret** is a head of the Department of Bioanalytical Instrumentation at the Institute of Analytical Chemistry and the group leader at CEITEC – Central European Institute of Technology in Brno, CR. His main research interests include capillary separations, laser based detection, miniaturization and mass spectrometry coupling. Besides working as a senior deputy editor of Electrophoresis, he is also an Associate Director of CASSS – an International Separation Science Society and member of the Learned Society of the Czech Republic.



**András Gelencsér** has graduated from the Veszprém University of Chemical Engineering in 1990, and received his PhD degree in the field of Environmental Sciences in 1995 and DSc in 2002. He has been the head of the Air Chemistry Research Group of the Hungarian Academy of Sciences since 2005. He became full time professor at the University of Pannonia in 2006 of which he has been the rector since 2015.

His main research area is atmospheric chemistry, in particular atmospheric aerosol chemistry related to air pollution and climate change. He was the first to demonstrate humus formation and establish the importance of biomass burning related coloured particles (brown carbon) in the absorption of sunlight in the global atmosphere. He authored a monograph entitled Carbonaceous Aerosol published by Springer in 2004. He has participated in several international projects and has a large number of joint publications with distinguished scientists. So far he has received more than 4200 independent SCI citations on his works. In 2016 he published a book on the impact of humanity on the global atmosphere in Hungarian. For his scientific achievements he was awarded an Order of Merit of the Hungarian Republic in 2014, Prima Primissima Award in 2016 and the Award of the Hungarian Academy of Sciences in 2017.



**Norberto Guzman** is currently Chief Scientific Officer-Director of Research at Princeton Biochemicals Inc., Princeton, New Jersey, U.S.A. Dr. Guzman's expertise is primarily in biomedicine and biotechnology with emphasis in protein biochemistry and immunochemistry. At present, his main research interest is in the determination of biomarkers of inflammatory processes.

Dr. Guzman received a B.Sc. degree in biochemistry (clinical biochemistry) from a Joint Undergraduate Program of the University of Concepcion and the University of Chile, Santiago, Chile; a M.Sc. degree in biochemistry (cell and molecular biology) from the Medical College of Georgia, Augusta, Georgia, U.S.A.; and a Ph.D. degree in biochemistry (protein biochemistry) from a Joint Graduate

Program of the University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School (formerly Rutgers Medical School) and Rutgers, The State University of New Jersey, New Brunswick, New Jersey, U.S.A. Dr. Guzman has worked in academic medical institutions, diagnostic and pharmaceutical companies, including Mount Sinai School of Medicine, Roche Diagnostic Systems, Hoffman-La Roche, and Johnson & Johnson.



**Svetlana M. Krylova** obtained her PhD from the Russian Academy of Sciences. She had over a decade of research leadership experience in the area of medical diagnostics and drug development in the biotechnological and pharmaceutical companies in Canada. She has been a contract faculty member at York University in Toronto since 2008. Dr. Krylova is also leading research projects in the area of Bioanalytical Chemistry as a Senior Research Associate in the Centre for Research on Biomolecular Interactions at York University.



**Sergey N. Krylov** is Professor, York Research Chair, and Director of Centre for Research on Biomolecular Interactions at York University in Toronto. He obtained his scientific degrees from Lomonosov Moscow State University and underwent postdoctoral training with Prof. H. Brian Dunford and Prof. Norman J. Dovichi at the University of Alberta. Dr. Krylov is recognized internationally for his pioneering work in the fields of chemical cytometry, kinetic analysis of affinity interactions, and methods for selection and applications of oligonucleotide aptamers. He recently became interested in development of innovative technologies for cancer subtyping and continuous-flow synthesis / purification / analysis. His research contributions have been recognized with a number of awards including Petro Canada Young Investigator Award, Premier's Research Excellence Award, President's Research Excellence Award (York University) and two recognitions from the Chemical Society of Canada: McBryde Medal and Maxxam Award.



**Ferenc Kilar** finished his studies in chemistry at Eötvös Loránd University, Budapest in 1977. After graduation he was working at the Institute of Enzymology, Budapest, and then he moved to University of Pécs, where he is working since 1983. He received his PhD (CSc) in 1986 and the degree of Doctor of Science in 1995. In 1997 he was appointed to be full professor as the Head of the Department of Analytical Chemistry and director of the Institute of Bioanalysis at Pécs. Since 2000 he is the Head of the Doctoral School in Chemistry. Between 1985 and 1992, he spent more than 5 years as visiting researcher at Uppsala University, Sweden, working on the development and application of capillary electrophoresis at the Department of Biochemistry. His research area covers protein-chemistry and the development and application of modern separation methods in bioanalysis. He is a co-author of

more than 150 scientific publications and 3 books. He is a member of several national and international research consortia and received several national and European grants. He was a visiting professor at Università "La Sapienza" and Istituto di Cromatografia, Rome, Italy, University of Bern, Switzerland and L'Institut Pasteur, Paris, France. He was the co-editor in chief of the Journal of Biochemical and Biophysical Methods (2001-2008), and member of the editorial boards of the Hungarian Chemical Journal (2001-2007), Studia Universitatis Babes-Bolyai Chemia (since 2007), Electrophoresis (since 2008), Journal of Proteomics (since 2008).



**Herbert Lindner** began his undergraduate education in the field of chemistry at the University of Innsbruck, Austria. In 1982, while employed as a Contract Assistant at the Institute of Organic and Pharmaceutical Chemistry, he obtained his Ph.D. Later in the same year, he relocated within the University to a position of Contract Assistant at the Department of Medical Chemistry and Biochemistry and, subsequently, was promoted to the position of University Assistant in 1984. In 1992, he received the award of Habilitation and "venialegendi" for Biochemistry. He was promoted to the position of Assistant Professor in 1994, and then to Associate Professor three years later. By 2007 Herbert Lindner was appointed Head of the Protein Micro-Analysis Facility at Innsbruck Medical University.

In the late 1980s he established a bioanalytical research group with focus on the development of high-resolution methods for the separation and identification of post-translational modified proteins to investigate their biological significance. Now, as a result of a continuous development program over many years, his group also offers a wide range of analytical methods and services to support the work of other research scientist in the University. The analytical tools developed and routinely applied in his laboratory led to numerous publications and successful national and international collaborations.



**Katalin Medzihradzky** is an Adjunct Professor of Pharmaceutical Chemistry in the School of Pharmacy of the University of California San Francisco, and the Head of the Laboratory of Proteomics Research in the Biological Research Centre of the Hungarian Academy of Sciences. She is an expert in protein analysis using mass spectrometry, especially in MS/MS data interpretation, de novo sequencing and studying post-translational modifications. She got her PhD in organic chemistry at the Eötvös Lóránd University in Budapest. She learned the basics of mass spectrometry in the Gedeon Richter LTD. Later she took a postdoc position in the Mass Spectrometry Facility of UCSF where she started to analyze proteins. Concurrently she has been conducting proteomics research in Szeged for 17 years by now. She is a head instructor of the Proteomics Course in Cold Spring Harbor Laboratory, and a member of the editorial board of Molecular and Cellular Proteomics. She has ~180 peer-reviewed publications.



**Marek Minarik** received his Ph.D. in bioanalytical chemistry from the Northeastern University in Boston in 2001 with Barry Karger at the Barnett Institute. The topic of his Ph.D. thesis was development of capillary-array electrophoresis instrumentation for micropreparative bioanalysis. Between 2000 and 2002 he worked in R&D at Molecular-Dynamics (later Amersham Biosciences) in Sunnyvale, CA developing applications for clinical and forensic DNA testing. He has authored over 50 scientific papers and 4 issued patents (3 US, 1 International). His main area of research interest is in development and application of tools and technologies for bioanalysis, mainly HPLC and CE separation techniques with main emphasis on clinical applications. Currently, he is a President and CEO of Genomac Research Institute in Prague, Czech Republic that he co-founded in 2001. Genomac is a private genomic research center focusing on screening and detection of molecular markers including liquid-biopsy technology for monitoring of cancer treatment and progression. Marek is also currently serving as a General Manager of Watrex Praha company in Prague, the leading manufacturer of HPLC instrumentation and columns in Eastern and Central Europe. Aside from commercial affiliations Marek currently holds an assistant professor position at the Department of Analytical Chemistry, Faculty of Sciences, Charles University in Prague lecturing on Genomic analysis in clinical practice.



**Pier Giorgio Righetti** earned his Ph. D. in Organic Chemistry from the University of Pavia in 1965. He then spent 3 years as a Post. Doc. at MIT and 1 year at Harvard (Cambridge, Mass, USA). He is now Emeritus Professor at the Milan's Polytechnic.

He is in the Editorial Board of Electrophoresis, J. Proteomics, BioTechniques, Proteomics, Proteomics Clinical Applications. He has co-authored the book Boschetti, E. Righetti, P.G. Low-Abundance Proteome Discovery; State of the Art and Protocols, Elsevier, Amsterdam, 2013, pp. 1-341.

He has developed isoelectric focusing in immobilized pH gradients, multicompartement electrolyzers with isoelectric membranes, membrane-trapped enzyme reactors, temperature-programmed capillary electrophoresis and combinatorial peptide ligand libraries for detection of the low-abundance proteome. On 560 articles reviewed by the ISI Web of Knowledge (Thomson Reuters), Righetti scores 22.200 citations, with an average of 39 citations/article and with a H-index of 67. During the years 2005-2013 he has received citations ranging from 1000 to 1200 per year. He has won the CaSSS (California Separation Science Society) award (October 2006), and the Csaba Horvath Medal award, presented on April 15, 2008 by the Connecticut Separation Science Council (Yale University). In 2011, he has been nominated honorary member of the Spanish proteomics society and in 2012 he has won the prestigious Beckman award and medal granted in February at the Geneva MSB meeting. In 2014, in October, in Madrid, he has been given the HuPO award for proteomic research and in November, in Atlanta, the American Electrophoresis Society award.



**Maria Fernanda Silva** is a Professor at The National University of Cuyo and Principal Researcher of National Council for Research (CONICET) in Argentina. Her group is focused on the study of the biological role of plant secondary metabolites and the development of methodologies aligned with the principles Green Analytical Chemistry for the extraction and determination of analytes of food and pharmaceutical interest. The outcomes of the research activities have been presented and recognized at national and international scientific meetings and are regularly published in peer-refereed journals. Her research has received support from the National Council for Research (CONICET), National Agency for Science and Technology (ANPCyT), and National University of Cuyo.



**Károly Vékey** has graduated in chemistry, and got his PhD degree at Eötvös University, Budapest, Hungary. His main research field is analytical- and physical- and biochemistry, and a foremost expert in mass spectrometry. He has been active both in fundamental studies and in practical applications. Structure determination of organic compounds; pharmaceutical, biomedical and clinical applications are in the forefront of his interest. Recently he has been active in proteomics, in particular analyzing protein glycosylation. He has developed a novel theory of mass spectrometry, focusing on energetic aspects. He has written over 200 publications, edited two books; his works have been cited over 4000 times. He is editor of the *Journal of Mass Spectrometry*, and board member of other scientific journals.



**Robert Weinberger** has been a consultant specializing in the field of High Performance Capillary Electrophoresis and related technologies since 1991. He is President of CE Technologies, Inc., a consulting firm specializing in contract research in HPCE. He taught the American Chemical Society Short Course entitled "Capillary Electrophoresis of Biomolecules" for 25 years and is the author of the textbook "Practical Capillary Electrophoresis". He has written around 100 Magazine articles in *American Laboratory* and is the author of over 50 technical publications, book chapters and patents in the fields of electrophoresis and liquid chromatography. He served as an expert witness in capillary electrophoresis patent litigation and provided expert opinions concerning the purity of potentially contaminated heparin samples. Food and drug contamination, dietary supplements and glycoprotein separations are recent areas of investigation.



**John R. Yates** is the Ernest W. Hahn Professor in the Department of Chemical Physiology and Molecular and Cellular Neurobiology at The Scripps Research Institute. His research interests include development of integrated methods for tandem mass spectrometry analysis of protein mixtures, bioinformatics using mass spectrometry data, and biological studies involving proteomics. He is the lead inventor of the SEQUEST software for correlating tandem mass spectrometry data to sequences in the database and developer of the shotgun proteomics technique for the analysis of protein mixtures. His laboratory has developed the use of proteomic techniques to analyze protein complexes, posttranslational modifications, organelles and quantitative analysis of protein expression for the discovery of new biology.

Many proteomic approaches developed by Yates have become a national and international resource to many investigators in the scientific community. He has received the American Society

for Mass Spectrometry research award, the Pehr Edman Award in Protein Chemistry, the American Society for Mass Spectrometry Biemann Medal, the HUPO Distinguished Achievement Award in Proteomics, Herbert Sober Award from the ASBMB, and the Christian Anfinsen Award from The Protein Society. He was ranked by Citation Impact, Science Watch as one of the Top 100 Chemists for the decade, 2000-2010. He was #1 on a List of Most Influential in Analytical Chemistry compiled by The Analytical Scientist 10/30/2013 and is on the List Of Most Highly Influential Biomedical Researchers, 1996-2011, European J. Clinical Investigation 2013, 43, 1339-1365. He has published 707 scientific articles with 54,000 citations and an H-index of 112.



## Abstracts of oral presentations – Invited speakers

### EMERGING ANALYTICAL TECHNOLOGIES AND NOWADAYS PHARMACEUTICAL DRUG PRODUCTS DEVELOPMENT CHALLENGES

BIANCA AVRAMOVIČH, PH.D.

Global Analytical Forum Leader,  
Head of Analytical Technologies Unit, GR&D, KFS ISRAEL  
Teva Pharmaceuticals Industries LTD

#### **Abstract**

The emerging hyphenated instrumentations (microscopies, spectroscopies, and separations) reveal new boundaries for seeing and controlling.

These new analytical instruments are more sophisticated having high rate scanning capabilities with the specificity of the measurement as the biggest advantage.

High informational productivity is achieved when numerous parameters are measured in one measurement. The effective data management became the new challenge of today and shortly we will discuss the information technologies architecture required to deal with this flux of information.

A number of case studies will emphasize the new scientific and regulatory challenges the pharmaceutical industry faces and the approaches taken when developing drug products as nasal sprays, oral solid dosages, inhalers, liposomes and nano particles based products.

Personalized pharmacy challenges in a regulated environment (as the household 3D printing approach) as well as the opportunities identified, will be discussed.

The “digital health” fast and innovative developments will be discussed while focusing on biomarkers, dynamic sensors and other “smart technologies” with digitalized, personalized treatments.

# ANALYSIS OF PROTEIN GLYCOLYSATION OF HIGH BIOMEDICAL IMPORTANCE BY CAPILLARY ELECTROPHORESIS AND ITS COMBINATION WITH MASS SPECTROMETRY

JEFF CHAPMAN

Senior Director CE Business Unit  
SCIEX, Brea, California, USA

## **Abstract**

There is a growing interest for rapid and high-resolution N-glycosylation analysis of glycoproteins for basic biomedical research especially in biomarker discovery. Key glycosylation functions include protein folding quality control and protein sorting. Cell surface glycans contain an array of antigenic epitopes, which bind to carbohydrate binding domains. These interactions are important in cell-cell and cell-pathogen interactions, also playing important roles in malignant transformation, so crucial in cancer research. Protein glycosylation happens via a serial heavily regulated biosynthetic steps in the endoplasmic reticulum and the Golgi apparatus. The final glycosylation structures is subject to factors including glycosyltransferase and glycosidase activities as well as nucleotide donor concentrations leading to large heterogeneity of glycoforms. To decipher these very complicated mixtures for better biochemical understanding, a user friendly and easy to use glycan release, fluorophore labeling and clean-up approach was developed resulting in 60 min sample preparation time using a novel magnetic bead mediated process with excellent yield and high reproducibility. The process can be readily automated, not necessitating any centrifugation and vacuum centrifugation steps, thus easily applicable in routine clinical laboratories. The prepared samples are separated by high performance capillary electrophoresis with laser induced fluorescent detection (CE-LIF) that provides migration time-based identification of complex carbohydrates based on a glucose unit (GU) database. Coupling with mass spectrometry, CE offers additional structural verification options. Integration of CE and electrospray ionization (ESI) into a single dynamic process (CESI) provides the capability of performing CE separation and MS ionization with ultra-low flow rates, resulting in reduced ion suppression and improved sensitivity. Examples of glycosylation analysis by CE-LIF and CESI-MS methods of formalin fixed paraffin embedded (FFPE) samples and circulating tumor cell surface (CTC) glycosylation will be thoroughly discussed.

# EFFECTS OF METAL IONS, ALKYLAMINES AND ORGANIC SOLVENTS ON THE SEPARATION OF BIOMOLECULES BY CAPILLARY ELECTROPHORESIS

DANILO CORRADINI

Research Director, Italian National Research Council,  
Rome, Italy

## **Abstract**

The composition of the background electrolyte solution (BGE) employed in capillary zone electrophoresis (CZE) can strongly influence sample solubility and detection, native conformation of biopolymers, molecular aggregation, electrophoretic mobility and the electroosmotic flow (EOF). Consequently, selecting the proper composition of BGE is of paramount importance in optimizing the separation of the analytes in CZE. The appropriate selection of the buffer requires evaluating the physical-chemical properties of all components of the buffer system, including buffering capacity, conductivity, and compatibility with the detection system and with the sample.

This communication discusses theoretical and practical aspects of the influence of the composition of the BGE on both the separation behaviour of biomolecules and the generation of EOF in bare fused-silica capillaries employed in CZE. Secondary equilibrium in solution between ionogenic analytes and components of the BGE can drastically affect the selectivity, which is based on differences in the electrophoretic mobility of the analytes, which depend on their effective charge-to-hydrodynamic radius ratios. This implies that selectivity is strongly influenced by the pH of the BGE as well as by the presence of suitable counterions and other interacting agents that may affect the net charge or/and the hydrodynamic radius of the analytes. The talk includes the discussion on the use of BGEs appositively tailored for separating proteins, peptides and other biomolecules by capillary electrophoresis with bare fused-silica capillaries. Most of the investigated solutions consist of buffering agents having the capability of controlling the protonic equilibrium in a wide pH range while preventing the undesirable interactions between basic analytes and the inner surface of bare fused-silica capillaries. Also discussed is the use of organic solvents and suitable additives incorporated into the BGEs traditionally employed in CZE.

# ANALYTICAL METHODOLOGIES IN THE SEARCH OF GLYCOBIOMARKERS

MERCEDES DE FRUTOS<sup>1\*</sup>, ANGEL PUERTA<sup>1</sup>, DIANA NAVARRO-CALDERON<sup>1</sup>, NOEMI FARINA-GOMEZ<sup>1</sup>,  
ANDRAS ACS<sup>2,3</sup>, EDUARDO ALBERS-ACOSTA<sup>4</sup>, CARLOS OLIVIER<sup>4</sup>, LASZLO DRAHOS<sup>2</sup>, KAROLY VEKEY<sup>2</sup>, AND  
JOSE CARLOS DIEZ-MASA<sup>1</sup>

<sup>1</sup> Department of Instrumental Analysis, Institute of Organic Chemistry (IQOG-CSIC), Madrid, 28006, SPAIN

<sup>2</sup> MS Proteomics Research Group, Research Centre for Natural Sciences, Hungarian Academy of Sciences,  
Budapest, 1117, HUNGARY

<sup>3</sup> Semmelweis University, Budapest, 1085, HUNGARY

<sup>4</sup> Department of Urology, Hospital La Princesa, Madrid, 28006, SPAIN

## Abstract

Proteoforms of glycoproteins, which are the result of different modifications such as genetic variants, changes in glycosylation and other PTMs of the protein, are very attractive in the search of biomarkers. In the living beings, their pathophysiological conditions determine what of these heterogeneities take place. Therefore, detecting quali- or quantitative differences in the proteoforms can be useful for finding disease biomarkers. Different approaches to detect these changes include from studying the monosaccharides, glycans, or glycopeptides until studying the intact non-hydrolyzed glycoprotein. For each approach several analytical techniques have been developed and are constantly improved.

In this talk, the studies that we perform to detect changes happening in intact glycoproteins (AGP and PSA) as potential biomarkers of vascular diseases and of prostate cancer will be shown. Special attention will be paid to the immunochromatographic sample preparation step needed to analyze the target glycoproteins in biofluids. Emphasis will be made on the analyses of the intact glycoproteins performed by capillary electrophoresis. In addition, the nanoLC-MS/MS study being performed for analysis of PSA glycopeptides will be shown.

**Acknowledgments:** MINECO (grant CTQ2013-43236R) and the contract in frame of Youth Guarantee Implementation Plans financed by ESF and YEI (D. N.-C.).

# TO INTERFACE OR NOT TO INTERFACE IN CE-MS. ONE STORY, TWO POINTS OF VIEW

FRANTISEK FORET, ANNA TYCOVA, JANA KRENKOVA, KAREL KLEPARNIK

Institute of Analytical Chemistry AS CR, v.v.i., Brno, 602 00, Czech Republic

## Abstract

Capillary electrophoresis coupled to mass spectrometry (CE/MS) is gaining its space among the most powerful tools in modern (bio)analytical laboratory. The most challenging instrumental aspect in CE/MS is striking the balance between the stability and reproducibility of the signal and sensitivity of the analysis. Several interface designs have been developed in the past decade addressing the variety of instrumental aspects and ease of operation. Most of the interfaces can be categorized either into the sheath flow arrangement (considered to be a de facto standard), or a sheathless arrangement, often expected to provide the ultimate sensitivity. In this work we have explored two, application dependent, approaches. A hybrid design, utilizing a microfabricated liquid junction/sprayer module, allowed coupling to a commercial, fully automated, CE analyser equipped with standard (50-75  $\mu\text{m}$  ID) separation capillaries. In the second, “interface-free” approach, the CE/MS analysis was performed in a narrow bore (<20  $\mu\text{m}$  ID) separation capillary forming one entity with the electrospray tip. The high voltage, applied at the injection end of the capillary, served for both the separation and electrospray ionization. Optimum conditions for the separation and electrospray ionization were achieved with voltage programming. The performance of both CE/MS systems was tested for separations of metabolites, peptides and glycoproteins and compared to other designs with respect to the separation efficiency, sensitivity and sample consumption.

## References:

- [1] Tycova, A.; Vido, M.; Kovarikova, P.; Foret, F. Interface-free capillary electrophoresis-mass spectrometry system with nanospray ionization—Analysis of dexrazoxane in blood plasma. *J.Chromatogr.*, 2016, 1466, 173-179.
- [2] Krenkova, J.; Kleparnik, K.; Grym, J.; Luksch, J.; Foret, F.: Self-aligning subatmospheric hybrid liquid junction electrospray interface for capillary electrophoresis. *Electrophoresis* 2016, 37, 414–417.

# BRIDGING SCALES AND DISCIPLINES: SOOT NANOPARTICLES IN THE ATMOSPHERE

ANDRÁS GELENCSÉR

Institute for Environmental Sciences, University of Pannonia, Veszprém, H-8200, HUNGARY

## **Abstract**

Soot particles are among the most deadly and at the same time the most powerful climate-modifying pollutants ever produced by mankind. Soot nanoparticles that form in high-temperature combustion have the potential to translocate across the epithelium into the bloodstream. Their small size, resilience, high specific surface area loaded with adsorbed carcinogenic and mutagenic compounds make them particularly harmful to human health. Each year 10 million metric tons of soot particles are leaking into the atmosphere, an amount that would instantly kill off most life on earth should it be released at once [1]. However, the effect of this immense amount of soot on the Earth's climate is still devastating and today soot comes second after carbon dioxide in the magnitude of its climatic effect. Even when precipitated out of the atmosphere, dark soot particles modify snow albedo thus contribute to the accelerated melting of the Arctic sea ice and mountain glaciers. There is a firm scientific consensus about the necessity of instant and effective reduction of soot emissions that would be beneficial for both human health and climate, but with practically no effect.

## **References:**

[1] Bardeena, C. G.; Garcia, R. R.; Toon, O. B.; Conley, A. J.: On transient climate change at the Cretaceous–Paleogene boundary due to atmospheric soot injections, *Proc. Nat. Acad. Sci.* 2017 114 (36) 7415-7424 doi: 10.1073/pnas.1708980114

# **ADVANCEMENTS IN MINIATURIZED IMMUNOAFFINITY CAPILLARY ELECTROPHORESIS: SHIFTING PARADIGMS IN BIOANALYSIS TO MONITOR WELLNESS, DISEASE, AND TREATMENT EFFECTIVENESS**

NORBERTO A. GUZMAN

Department of Immunoengineering-Bioanalysis, Princeton Biochemicals, Inc, Princeton, USA

## **Abstract**

Point-of-care testing (POCT) is a rapid growing field with a potential key role for improving operational efficiency within healthcare settings, if correctly utilized. Specifically, the rapid delivery of accurate, sensitive, and specific results at the bedside has shown to facilitate clinical decision-making and may improve clinical outcomes in patient care. Ideally, POCT should be one of the most efficient and economical tools for medical management. However, there are limitations to consider before traditional diagnostic testing can uniformly transition to POCT.

In this presentation, I will describe a novel instrument based on a two-dimensional technology, named immunoaffinity capillary electrophoresis (IACE), which can address many of these limitations associated with POCT. IACE utilizes immunoaffinity-capture techniques found in some immunoassays, such as ELISA, paired with a high-resolution analytical separation technique, such as capillary electrophoresis. Distinctively, this promising instrument yields no false-positive or false-negative results and can be a great asset in both resource-limited and large private healthcare settings. Furthermore, while inpatient and outpatient test results produced by this technology can be interpreted bedside, by a team of healthcare professionals, I anticipated that home test results can be linked via a secured Internet connection to clinical facilities equipped with medical data banks and staffed by physicians for interpretation and follow up, as necessary.

In summary, I foresee that this POCT technology will have tremendous implications for healthcare improvement both on an individual and global scale, in particular to monitor wellness, disease and treatment effectiveness. I will describe the details, applications, and potential impact of this technology.

# CAPILLARY ELECTROPHORESIS-BASED ANALYSIS OF ENZYMATIC MODIFICATIONS OF PROTEINS AND DNA

SVETLANA M. KRYLOVA,<sup>1\*</sup> AGNESHA SHALA-LAWRENCE,<sup>1</sup> JAMES L. HOUGLAND,<sup>2</sup> CHRISTOPHER J. SCHOFIELD,<sup>3</sup> SERGEY N. KRYLOV<sup>1</sup>

<sup>1</sup> Department of Chemistry and Centre for Research on Biomolecular Interactions, York University, Toronto, Ontario M2N 3P5, CANADA

<sup>2</sup> Department of Chemistry, Syracuse University, Syracuse, New York 13244, United States

<sup>3</sup> Oxford Centre for Integrative Systems Biology, University of Oxford, Oxford, OX1 3TA, United Kingdom

## Abstract

Enzymatic modifications of proteins and DNA are pivotal to cellular regulation. In particular, protein lipidation plays a unique and essential role in regulating protein localization and trafficking within the cell by directly mediating protein-membrane interactions. One central challenge in studying protein lipidation has been the difficulty in measuring the distribution and relative populations of precursor and chemically modified forms of proteins. The lack of quantitative methods that could simultaneously measure the amounts of both protein forms has complicated quantitative analysis of the regulation, functional impact, and biological requirement for protein lipidation. To date, analytical methods used to study protein lipidation have focused primarily on identifying lipidated proteins. In exploring analytical methods for efficiently separating and detecting both the prenylated and non-prenylated forms of a protein, we used fluorescent reporter proteins (eGFP and TagRFP) which have been designed to become substrates of protein farnesyltransferase (FTase) and protein geranylgeranyltransferase type I (GGTase-I). A similar problem of separating a relatively large biological polymer-based substrate and the product exists in quantitation of enzymatic modifications of DNA. Our models for this type of modifications were based on several 2-oxoglutarate (2OG)-dependent oxygenases that remove a methyl group from a methylated DNA base. Such demethylation processes are important for protection of genomic DNA from damage induced by alkylation agents. The use of biologically relevant DNA substrates of (2OG)-dependent oxygenases faces a challenge of quantitatively distinguishing them from their products, which differ only by few methyl groups. In both types of enzymatic reactions catalyzing protein and DNA modifications, the simultaneous quantitation of both the substrate and the product can be achieved *via* their separation by capillary electrophoresis (CE) followed by detection with laser-induced fluorescence (LIF). Both types of experimental models to study enzymatic modifications of proteins and DNA relied on choosing a fluorescently-labeled biologically relevant substrate, and using a surfactant (SDS) as a buffer additive to facilitate efficient separation of the substrate from the product of essentially the same size (different by a small chemical group). The described CE-separation-based approach



will be useful for understanding roles of enzymatic modifications of proteins and DNA in cellular regulation and for finding enzyme inhibitors, studying mechanisms of inhibition, and discovery of drug leads [1-4].

### **References:**

- [1] Shala-Lawrence, A.; Decker, M.J.; Krylova, S.M.; Gangopadhyay, S.S.; Beloborodov, S.S.; Hougland, J.L.; Krylov, S.N.: Simultaneous analysis of non-lipidated protein and its lipidated counterpart: enabling quantitative investigation of protein lipidation's impact on cellular regulation, *Angew. Chem. Int. Ed.*, submitted
- [2] Krylova, S.M.; Koshkin, V.; Bagg, E.; Schofield, C.J.; Krylov, S.N. Mechanistic studies on the application of DNA aptamers as Inhibitors of 2-oxoglutarate-dependent oxygenase, *J. Med. Chem.* 2012 55(7), 3546–3552
- [3] Woon, E.C.Y.; Demetriades, M.; Bagg, E.A.L.; Aik, W.S.; Krylova, S.M.; Ma, J.H.Y.; Chan, M.C.; Walport, L.J.; Wegman, D.W.; Dack, K.N.; McDonough, M.A.; Krylov, S.N.; Schofield, C.J. Dynamic combinatorial mass spectrometry leads to inhibitors of a 2-oxoglutarate dependent nucleic acid demethylase, *J. Med. Chem.* 2012 55(5), 2173-2184
- Cherney, L.T.; Krylov, S.N.: Theoretical estimation of drag tag lengths for direct quantitative analysis of multiple miRNAs (DQAMmiR), *Analyst* 2013 138 (2), 553–558
- [4] Krylova, S.M.; Karkhanina, A.A.; Musheev, M.U.; Bagg, E.A.L.; Schofield, C.J.; Krylov, S.N.: DNA aptamers for as analytical tools for the quantitative analysis of DNA-dealkylating enzymes, *Anal. Biochem.* 2011 414, 261–265

# PROSPECTIVES OF CAPILLARY ELECTROPHORESIS IN VALIDATION OF MOLECULAR BIOMARKERS OF CANCER

SERGEY N. KRYLOV,\* LIANG HU, MANSI ANAND, SVETLANA M. KRYLOVA, VASILIJ KOSHKIN

Department of Chemistry and Centre for Research on Biomolecular Interactions, York University, Toronto, Ontario M2N 3P5, CANADA

## Abstract

Effective treatment of cancer requires molecular biomarkers (characteristic molecules present in tumor cells) that can predict disease aggressiveness and its response to therapy. A large number of predictive biomarkers have been reported, but the following studies proved non-robustness of the majority of them. As a result, only a few biomarkers have been approved by FDA for clinical use. Biomarker non-robustness originates, in particular, from non-robustness of: (i) specimen collection, preservation, and storage, (ii) specimen recovery and processing (extraction, enzymatic modifications, labeling *etc.* of target molecules), and (iii) quantitative analysis of target molecules. Robustness of biomarkers can, thus, be improved *via*: reducing step 1 to specimen freezing, reducing step 2 to specimen thawing and cell lysis, and developing highly-robust assays applicable to crude cell lysates in step 3. Our research is motivated by an insight that capillary electrophoresis (CE) can serve as an analytical platform for robust analyses of molecular biomarkers applicable to crude cell lysates. To prove this hypothesis, we are creating a CE-based Technology for Analysis of miRNA Signatures (TAmiRS). In the past 7 years, we have developed TAmiRS into a practical technology with proven analytical validity, and we are now advancing TAmiRS to tests of its analytical and clinical utility [1–9]. In this lecture, the concept of TAmiRS and its analytical features will be explained; its advantages and limitation in comparison to other technologies will be pointed out. Conclusions will be made with regards to the generalization of CE-based approach for validation of molecular biomarkers of cancer.

## References:

- [1] Krylova, S.M.; Wegman, D.; Krylov, S.N.: Making DNA hybridization assays in capillary electrophoresis quantitative, *Anal. Chem.* 2010 82(11), 4428–4433
- [2] Wegman, D.W.; Krylov, S.N.: Direct quantitative analysis of multiple miRNAs (DQAMmiR), *Angew. Chem. Int. Ed.* 2011 50(44), 10335–10339
- [3] Cherney, L.T.; Krylov, S.N.: Theoretical estimation of drag tag lengths for direct quantitative analysis of multiple miRNAs (DQAMmiR), *Analyst* 2013 138 (2), 553–558
- [4] Wegman, D.W.; Krylov, S.N.: Direct miRNA-hybridization assays and their potential in

diagnostics, *TrAC* 2013 44, 121–130

[5] Wegman, D.W.; Cherney, L.T.; Yousef, G.; Krylov, S.N.: Universal drag tag for direct quantitative analysis of multiple microRNAs, *Anal. Chem.* 2013 85(13), 6518–6523

[6] Ghasemi, F.; Wegman, D.W.; Kanoatov, M.; Yang, B.B.; Liu, S.K.; Yousef, G.M.; Krylov, S.N.: Improvements to direct quantitative analysis of multiple microRNAs facilitating faster analysis, *Anal. Chem.* 2013 85(21), 10062–10066

[7] Wegman, D.W.; Ghasemi, F.; Khorshidi, A.; Yang, B.B.; Liu, S.K.; Yousef, G.M.; Krylov, S.N.: Highly-sensitive amplification-free analysis of multiple miRNAs by combining capillary isotachopheresis and electrophoresis, *Anal. Chem.* 2015 87(2), 1404–1410

[8] Wegman, D.W.; Ghasemi, F.; Stasheuski, A.S.; Khorshidi, A.; Yang, B.B.; Liu, S.K.; Yousef, G.M.; Krylov, S.N.: Achieving single-nucleotide specificity in direct quantitative analysis of multiple microRNAs (DQAMmiR), *Anal. Chem.* 2016 88(4), 2472–2477

[9] Hu, L.; Stasheuski, A.S.; Wegman, D.W.; Wu, N.; Yang, B.B.; Hayder, H.; Peng, C.; Liu, S.K.; Yousef, G.M.; Krylov, S.N.: Accurate miRNA analysis in crude cell lysate by capillary electrophoresis-based hybridization assay in comparison with quantitative reverse transcription-polymerase chain reaction, *Anal. Chem.* 2017 89(8), 4743–4748

# HOW TO DEVELOP CAPILLARY ELECTROPHORESIS?

FERENC KILÁR

University of Pécs, Pécs, Hungary

## **Abstract**

Steps in electrophoresis from Tiselius to CE(CE). Tasks and problems solved and unsolved in capillary electrophoresis. A personal view and experience in this separation area.

# ADVANCES IN THE SEPARATION OF CO- AND POST-TRANSLATIONAL PROTEIN MODIFICATIONS USING CE-MS

HERBERT H. LINDNER, KLAUS FASERL AND BETTINA SARG

Innsbruck Medical University, Biocenter, Division of Clinical Biochemistry  
Innrain 80, A-6020 Innsbruck, Austria

## **Abstract**

In higher eukaryotes a majority of proteins is post-translationally modified and these modifications are often essential for the function of a protein. The goal of PTM analysis is to localize the amino acid residues in a given protein that are altered in vivo to activate, inactivate, or modify the biological activity of proteins. Due to its biochemical importance, various analytical techniques for the characterization of modified peptides in complex mixtures generated by enzymatic digestion of multiple proteins have been described. Especially HPLC coupled with high-resolution mass spectrometry is a well-established technique and in many cases the method of choice for the separation and identification of proteins and their modification sites.

An alternative strategy, capillary electrophoresis coupled to mass spectrometry seems to be gaining momentum especially for the analysis of protein modifications. This work describes the use of low-flow CESI-MS/MS to determine post-translational modifications, e.g. acetylation, phosphorylation, methylation, deamidation and citrullination in medium complex nuclear samples. Further, the suitability of CE-MS for the analyses of intact nuclear proteins and their multiply modified forms was investigated to provide information regarding the extent of existing PTMs. Based on the results obtained CE-MS can be considered a complementary technique to conventional LC-MS and an alternative approach for PTM analysis.

# NEGLECTED POST-TRANSLATIONAL MODIFICATIONS: EXTRACELLULAR O-GLYCOSYLATION AND PHOSPHORYLATION.

KATALIN F. MEDZIHRADESKY<sup>1,2</sup>, EVA HUNYADI-GULYAS<sup>1</sup>, EVA KLEMENT<sup>1</sup>, ZSUZSA DARULA<sup>1</sup>

<sup>1</sup> Laboratory of Proteomics Research, Biological Research Centre of the Hungarian Academy of Sciences, Szeged, Hungary, H-6726

<sup>2</sup> Department of Pharmaceutical Sciences, School of Pharmacy, University of California San Francisco, San Francisco, USA, CA 94158

## **Abstract**

Many proteomic studies aim to describe the ‘normal’ composition of protein complexes, cell organelles, tissues or body fluids, and then to detect the changes occurring as the result of certain stimuli, or diseases. Such research produced impressive results, but we still have limited knowledge about the occurrence and changes of post-translational modifications (PTMs) that most certainly are significant players in the regulation of complex-formation, protein-protein interactions, and biological activity. In addition, PTMs that modify proteins located in the extracellular space have been neglected for a long time despite the ever growing evidence that i) they are really important for our wellbeing ii) they may influence/control/regulate intracellular processes.

Our research focuses on two quite ‘elusive’ extracellular PTMs, the phosphorylation and glycosylation of Ser, Thr and Tyr residues. They are exceptionally hard to tackle, but their biological role(s) – at least what we know about them – are really exciting. We are developing protocols for the enrichment of modified peptides from readily available body fluids and then use reversed phase nanochromatography linked to high sensitivity tandem mass spectrometry to analyze the resulting mixtures. We are gathering data on the proteins and the sites modified, and we do hope that our basic research project will lead to translational results.

# CANCER DIAGNOSIS AND MONITORING BY CE-BASED LIQUID BIOPSY ASSAY

MAREK MINARIK

Centre for applied genomics of solid tumors (CEGES), Genomac Research Institute  
Prague, Czech Republic

## **Abstract**

The liquid biopsy is gradually becoming inherent part of diagnosis and treatment of many solid tumors. The technique is based on detection of specific tumor-derived elements in peripheral blood circulation [1]. The main effort has been devoted to study of two main entities, namely the circulating tumor cells (CTC) and circulating tumor DNA (ctDNA). While CTCs offer more complex view of the tumor including information on RNA and protein expression, analysis of ctDNA is far less demanding on dedicated expensive instrumentation and currently is mainly done by adapted mutation detection techniques.

In 2012 we have demonstrated application of denaturing capillary electrophoresis (DCE) for detection of ctDNA in patients with colorectal cancer undergoing surgical treatment [2]. Since then we have extended the spectrum of applications for this CE-based liquid-biopsy assay to other solid cancers including lung cancer, pancreatic cancer, stomach cancer and head and neck cancer. Our rates of ctDNA positive patients correspond to the mutation sensitivity level of 0.1%. In order to match the current sensitivity of the contemporary costly digital liquid biopsy assays, we have recently developed a modified protocol adopting an additional mutant enrichment step using an experimental micropreparative DCE system. This has resulted in an improvement of sensitivity to 0.01%.

The current presentation will present utility of the assay in clinical practice and summarize the most relevant results and interesting cases. Supported by Ministry of Health of the Czech Republic grant no. AZV 15-27939A.

## **References:**

- [1] Crowley E, Di Nicolantonio F, Loupakis F, Bardelli A. Liquid biopsy: monitoring cancer-genetics in the blood. *Nat Rev Clin Oncol.* 2013, 10, 472-484.
- [2] Levy, M., Benesova, L., Lipska, L., Belsanova, B., Minarikova, P., Veprekova, G., Zavoral, M., Minarik, M., Utility of Cell-free Tumour DNA for Post-Surgical Follow-up of Colorectal Cancer Patients, *Anticancer Res.* (2012), 32, 1621-1626.

# TWO MINIATURIZED MINI-SENSORS: FOR FORMALDEHYDE FUMES AND FOR GLUCOSE ANALYSIS

GLEB ZILBERSTEIN<sup>1</sup>, ROMAN ZILBERSTEIN<sup>1</sup>, URIEL MAOR<sup>1</sup>, PIER GIORGIO RIGHETTI<sup>2\*1</sup>

<sup>1</sup> Spectrophon Ltd, Rehovot, ISRAEL

<sup>2</sup> Department of Chemistry, Politecnico di Milano, Via Mancinelli 7, Milano 20131, ITALY

## Abstract

The formaldehyde (FA) sensor is used to monitor fumes in the atmosphere, and is based on the redox reaction between FA and silver nitrate. The sensor is worn as a bracelet and the data acquired are transferred via a blue-tooth channel to a smartphone. A dedicated software transforms the signal from a grey to a colour scale. The signal response has been assessed over low (20 to 120 ppb) as well as higher (1-15 ppm range) levels. The sensor has been applied to monitor potential FA fumes of some artwork in the Summer Palace in Beijing and the modifications induced by FA treatment on a precious Stradivarius violin. Monitoring of FA fumes in different work environment has shown that the greatest levels are found in beauty saloons when using fingernails lacquer!

A non-invasive mini-sensor for blood glucose concentration assessment has also been developed. The monitoring is performed by gently pressing a wrist or fingertip onto the chemochromic mixture coating a thin glass or polymer film positioned on the back panel of a smart watch. The various chemochromic components measure the absolute values of the following sweat metabolites: acetone, acetone beta-hydroxybutirate, aceto acetate, water, carbon dioxide, lactate anion, pyruvic acid, Na and K salts. Taken together, all these parameters give information about blood glucose concentration, calculated via multivariate analysis based on neural network algorithms built into the sensor. The Clarke Error Grid shows an excellent correlation between a standard invasive glucose analyser and the present non-invasive sensor, with all points aligned along a 45 degree diagonal and contained only in sector A. Graphs measuring glucose levels five times a day, for different individuals (male and female) show a good correlation between the two curves of conventional, invasive meters vs. the non-invasive sensor, with an error of ca. 15%.



# NOVEL APPROACHES MEDIATED BY TAILOR-MADE GREEN SOLVENTS FOR THE CHARACTERIZATION OF BIOLOGICALLY ACTIVE COMPOUNDS

M, DE LOS A. FERNANDEZ<sup>1</sup> FEDERICO GOMEZ,<sup>1</sup> MAGDALENA ESPINO<sup>1</sup> AND M, FERNANDA SILVA<sup>3</sup>

<sup>1</sup> Institute of Agricultural Biology (IBAM CONICET), Faculty of Agronomic Sciences, National University of Cuyo, Mendoza, 5505, ARGENTINA

## Abstract

Developing sustainable solvents is perhaps the most active area of Green Chemistry. Ionic liquids (ILs) and deep eutectic solvents (DES) have been revealed as a good option to replace harsh organic solvents. However, current ILs and DES have still some limitations to be applied to a real green industry. When the compounds that constitute the DES are primary metabolites, namely, aminoacids, organic acids, sugars, or choline derivatives, the DES are called Natural Deep Eutectic Solvents (NADES). NADES fully represent green chemistry principles [1]. These solvents offer many striking advantages including biodegradability, sustainability, low costs and simple preparation. In this communication, the recent contributions of our group regarding NADES with special emphasis on their chemometrical design, physico chemical characterization by NOESY NMR and molecular modelling, and analytical applications that utilize CE, ME and UPLC will be presented. Phenolic compounds as well as indoleamines have been determined in medicinal plants, wines, and different agro-food industrial by-products [1, 2]. Further perspectives of these truly green solvents in health-related areas such as pharmaceuticals, foods, and cosmetics will be described.

## References:

- [1] Fernandez, M., Espino, M. Gomez, F., Silva, M: Novel approaches mediated by tailor-made green solvents for the extraction of phenolic compounds from agro-food industrial by-products, *Food Chem* 2018 239, 671–678.
- [2] Gomez, F., Espino. M., Fernandez, M., Raba, J., Silva, M.: Enhanced electrochemical detection of quercetin by Natural Deep Eutectic Solvents, *Anal Chim Acta*, 2016, 936, 91-96.

# PROTEIN GLYCOSYLATION AND MASS SPECTROMETRY

KÁROLY VÉKEY

Hungarian Academy of Sciences, Research Centre for Natural Sciences  
Budapest, Hungary

## **Abstract**

The lecture will give an overview of mass spectrometric techniques for analysis of glycoproteins. The main points will be illustrated by practical examples.

# FOUR DECADES OF CAPILLARY ELECTROPHORESIS

ROBERT WEINBERGER

CE Technologies, Inc,  
New York, USA

## **Abstract**

While the pioneering work of Stellan Hjerten<sup>1</sup> set the stage for the development of modern CE, this story will begin in the 1980s with the reports of Jorgenson and Lukacs<sup>2</sup>. That decade was an age of discovery as the attributes of CE were determined. Separations of urinary porphyrins are used to describe the learning curve<sup>3</sup>. The 1990s was the decade of high aspirations. Few small molecules escaped separation and the foundation for the human genome project was developed. The first genome was sequenced by 2001. The advent of ultra-performance liquid chromatography (UPLC) introduced in 2004 negated the advantages of CE for small molecules. There were more disappointments when the CE separation of heparin and impurities<sup>4</sup> was replaced by an inferior anion-exchange method. The status of CE in the 2010s is alive and well. CE is generally accepted for biotechnology derived proteins using size separations, isoelectric focusing and for carbohydrates. CE mass spectrometry is beginning to mature and a variety of DNA and RNA applications are mature. Sanger sequencing is still important, primarily for checking plasmid integrity. The most important DNA application is for forensic human identification<sup>5</sup>. Clinical chemistry applications with emphasis on serum protein<sup>6</sup> and hemoglobin separations will be discussed.

## **References:**

- [1] Hjerten, S. (1967). "Free Zone Electrophoresis." *Chromatogr. Rev.* 9: 122-219.
- [2] Jorgenson, J. W. and K. D. Lukacs (1981). "Free-Zone Electrophoresis in Glass Capillaries." *Clin. Chem.* 27: 1551-1553.
- [3] Weinberger, R., E. Sapp, et al. (1990). "Capillary electrophoresis of urinary porphyrins with absorbance and fluorescence detection." *J Chromatogr* 516(1): 271-285.
- [4] Wielgos, T., K. Havel, et al. (2009). "Determination of impurities in heparin by capillary electrophoresis using high molarity phosphate buffers." *J Pharm Biomed Anal* 49(2): 319-326.
- [5] Butler, J. M., E. Buel, et al. (2004). "Forensic DNA typing by capillary electrophoresis using the ABI Prism 310 and 3100 genetic analyzers for STR analysis." *Electrophoresis* 25(10-11): 1397-1412.

[6] Bossuyt, X. (2003). "Separation of serum proteins by automated capillary zone electrophoresis." *Clin Chem Lab Med* 41(6): 762-772.

**COMPARATIVE N-GLYCOSYLATION ANALYSIS OF A BIOSIMILAR AND ITS INNOVATOR PRODUCT BY CE-LIF**

BEÁTA BORZA,<sup>1,2</sup> MÁRTON SZIGETI,<sup>1,2</sup> LÁSZLÓ HAJBA,<sup>2</sup> ÁKOS SZEKRÉNYES<sup>1,4</sup> AND ANDRÁS GUTTMAN<sup>1,2,3</sup>

1 Horváth Csaba Memorial Institute of Bioanalytical Research, University of Debrecen

2 MTA-PE Translational Glycomics Research Group, Research Institute for Biomolecular and Chemical Engineering, University of Pannonia, Veszprem, Hungary

3 SCIEX Separations, Brea, CA, USA

4 mAbxience Research, Parque Tecnológico de León, Julia Morros s/n 24009 Armunia, León, Spain

**Abstract**

There is a growing demand in the biopharmaceutical industry for recombinant protein pharmaceuticals such as monoclonal antibody-based therapeutics, hormones, growth factors, blood products, and recombinant vaccines. These biologics have played a progressively significant role in modern pharmaceuticals. Due to the expiration of several patents and regulatory data protection of original biotherapeutics mostly the recombinant monoclonal antibodies, competing companies have the opportunity to evolve their own biosimilar replica. Biosimilars are similar but not accurately the same as the innovator products. As a result of the limited information on manufacturing procedures, the developed process may contain some differences such as employed cell line, advanced cell culture conditions as well as purification processes. Consequently, there is crucial to find an appropriate analytical method, which is able to compare the final and the original therapeutics. Structural differences could affect their clinical performance. Thus, the comparison of the reference product and the biosimilar medicine are based on the following criteria: 1) primary structures; 2) higher-order structures (HOS); 3) posttranslational modifications; 4) degradation hotspots; 5) chemical modifications. For the comprehensive description it is recommended to use orthogonal analytical methods such as capillary electrophoresis (CE). Major of recombinant proteins (e.g. monoclonal antibodies) and their biosimilar versions are glycosylated. Glycosylation of biosimilar products could have a considerable influence on the pharmacokinetic (PK) profile, biological activity, serum half-life, and effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), and immunogenicity of the drug products. Thus, glycosylation is a critical quality attribute, that could be investigated with capillary electrophoresis.

**Acknowledgment**

The authors gratefully acknowledge the support of the Momentum (Lendület) grant # 97101 of the Hungarian Academy of Sciences (MTA-PE Translational Glycomics) and the National Research, Development and Innovation Office (NKFIH) (K 116263) grants of the Hungarian Government. This work was also supported by the BIONANO\_GINOP-2.3.2-15-2016-00017 project.

# ON-CAPILLARY DERIVATIZATIONS OF AMINO ACIDS IN CAPILLARY ELECTROPHORESIS USING NAPHTHALENE-2,3-DICARBOXALDEHYDE/SODIUM CYANIDE

ANDREA CELÁ, ALEŠ MÁDR AND ZDENĚK GLATZ\*

Department of Biochemistry, Faculty of Science, Masaryk University, Kamenice 5, Brno, 625 00, Czech Republic

**Abstract** – Automatic on-capillary derivatization of amino acids using naphthalene-2,3-dicarboxaldehyde/sodium cyanide as derivatization reagents were performed. Separately injected reactants were mixed both by transverse diffusion of laminar flow profiles (TDLFP) and electrophoretically mediated microanalysis (EMMA). Both mixing approaches were individually optimized and compared in terms of efficiency of the mixing, sensitivity, repeatability and applicability to various biological samples. The EMMA-based method provides up to 12× higher sensitivity and proved to be more robust to the sample composition compared with TDLFP-based method.

**Keywords:** capillary electrophoresis, fluorescence detection, on-capillary derivatization, amino acids, naphthalene-2,3-dicarboxaldehyde

## 1. Introduction

Amino acids (AAs) are metabolites of great importance to living organisms. They are not only building blocks of proteins, but they are involved in cell signalling and gene expression as well. Consequently, actual levels of AAs in biological fluids can reveal complex physiological state of an organism [1].

The most sensitive determination of AAs can be achieved by laser-induced fluorescence detection (LIF). Unfortunately, AAs exhibit low or no native fluorescence thus derivatization reaction is required to add fluorescence properties to AAs. Products of these reactions can be unstable and therefore proper standardization of the analytical procedure is necessary. Moreover, the derivatization reaction are often time consuming, laborious and derivatization reagents are relatively expensive.

Capillary electrophoresis (CE) offers unique way how to perform chemical or biochemical reactions without any special instrumental modification. This approach is called on-capillary [2] and is characterized by performing the reactions within the separation capillary just prior the separation. The inner space of the capillary is used both for the reaction and then for the separation as well. This approach can be fully automated and requires only minimum volumes of a sample and other reagents.

Generally, the sample and the reagents are injected into the capillary sequentially and then they are mixed within the capillary to form reaction mixture. Time of the reaction is strictly defined by the time

from the mixing to the application of the separation voltage. As a result, standardization of time-dependent product degradation is assured.

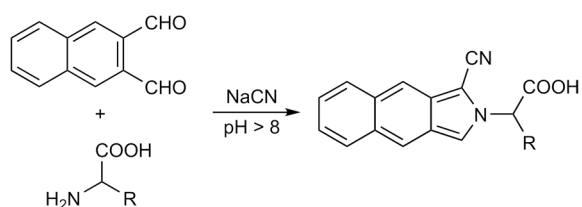
The mixing of the reactants in such a limited space requires unconventional methods of the mixing. There are generally two applicable mixing principles. The first is based on thermodynamic movements of particles (diffusion), whereas the second rely on electrophoretic movements of charged particles. Diffusion-based mixing is divided into two groups according to the direction of diffusion, which is responsible for the mixing. *At-inlet reactions* are characteristic with mixing by longitudinal diffusion, and mixing by transverse diffusion is called *transverse diffusion of laminar flow profiles* (TDLFP). *Electrophoretically mediated microanalysis* (EMMA) is a name of a group of mixing modes based on electromigration [2].

Each mixing approach has its advantages and disadvantages. At-inlet requires injection of very short plugs and is limited to only two separately injected reactants. TDLFP is considered to be generic and it is applicable to more than two separately injected reactants, however TDLFP provides limited overlap of even long plugs. Prerequisites of EMMA are different electrophoretic mobilities of reactants. EMMA provides superior mixing efficiency due to efficient overlap of long injected plugs; however, optimization of EMMA for more than two reactants is challenging and the developed method is not generic.

---

\*Correspondence: glatz@chemi.muni.cz

Naphthalene-2,3-dicarboxaldehyde (NDA) is the fluorogenic reagent with excitation/emission wavelengths 420/490 nm and provides thermally stable and highly fluorescent AAs derivatives at room temperature [3]. Due to a low cost, rapid reaction and solubility of NDA derivatives, NDA was employed in our experiments. NDA reacts in the presence of cyanide anion with a primary amino group to form highly fluorescent *N*-substituted 1-cyanobenz[*f*]isoindoles (CBIs). The scheme of the derivatization reaction is depicted at *Figure 1*.



*Figure 1*. Scheme of the derivatization of AA with NDA in the presence of NaCN yielding AA-CBIs solution was adjusted to pH 9.00 by addition of NaOH on pH-meter Orion 370 using Orion™ ROSS™ Sure-flow™ pH electrode (Thermo Fisher Scientific, Waltham, MA, USA). Adjusted solution was quantitatively transferred to a volumetric flask containing the appropriate weight of HP-β-CD and the appropriate volume of 1P. Then the solution was brought to the calibration mark of the volumetric flask by DI water giving BGE comprised of 135 mM H<sub>3</sub>BO<sub>3</sub>/NaOH, 73 mM SDS, 0.5 mM HP-β-CD and 6.7 % (v/v) 1P. BGE was thoroughly mixed, filtered using 0.45 μm polyamide membrane filter (Fisher Scientific, Pardubice, Czech Republic) and degassed in an ultrasonic bath (Branson Ultrasonic, Danbury, CT, USA) for 10 minutes. BGE was stored at laboratory temperature and used within a week.

## 2. Experimental

### 2.1. Chemicals

All chemicals were of analytical grade purity and were used without any purification. L-Alanine (Ala), L-alanyl-L-glutamine (Ala-Gln), L-arginine monohydrochloride (Arg), L-asparagine (Asn), L-aspartic acid (Asp), boric acid (H<sub>3</sub>BO<sub>3</sub>), L-cystine (Cyn), L-glutamine (Gln), L-glutamic acid (Glu), glycine (Gly), L-histidine monohydrochloride monohydrate (His), (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD), hydrochloric acid (HCl, 37%), L-isoleucine (Ile), L-leucine (Leu), L-lysine monohydrochloride (Lys), methanol (MeOH), L-methionine (Met), NDA, L-norvaline (nVal), L-phenylalanine (Phe), 1-propanol (1P), 2-propanol (IPA), sodium cyanide (NaCN), sodium dodecyl sulfate (SDS), L-serine (Ser), sodium hydroxide (NaOH), sulfuric acid (95%), taurine (Tau), L-threonine (Thr), L-tryptophan (Trp), Tween® 20, L-tyrosine (Tyr) and L-valine (Val) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized (DI) water (18.2MΩ at 25°C) was obtained by Direct-Q® 3 UV water purification system (Merck Millipore, Billerica, MA, USA).

### 2.2. Background electrolyte

The background electrolyte (BGE) was prepared by dissolving the appropriate weights of H<sub>3</sub>BO<sub>3</sub> and SDS in 80 % of final volume of DI water, the

### 2.3. Capillary treatment

Bare fused silica capillaries of 50 μm inner and 375 μm outer diameters were purchased from Polymicro Technologies (Phoenix, AZ, USA). Total length of the capillary was 66 cm and polyimide layer was removed by boiling sulfuric acid at the distance 45 cm from the anodic end of the capillary (effective length). Each newly cut capillary was rinsed at 50 °C by DI water, 1 M NaOH, 0.1 M NaOH, DI water for 5, 20, 10 and 10 minutes, respectively. Before analysis, the capillary was rinsed at 25 °C with 1 M NaOH, 0.1 M NaOH, DI water and BGE for 2, 1, 2 and 3 minutes, respectively. After analysis, the capillary was rinsed at 25 °C with DI water, 1%

(w/w) Tween® 20 and DI water for 1, 2 and 2 minutes, respectively.

#### 2.4. CE-LIF method

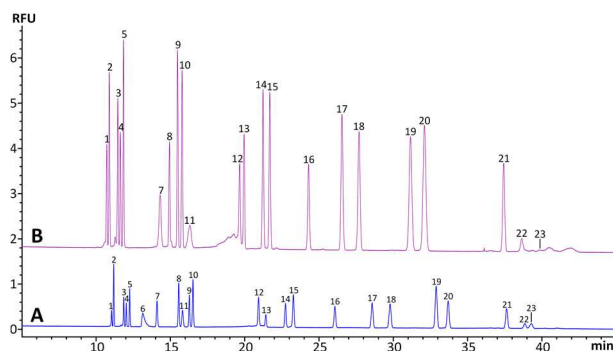
The Agilent G7100A CE System (Agilent Technologies, Waldbronn, Germany) coupled with external collinear LIF detector ZETALIF™ LED 480 (Picometrics, Labège, France) using excitation/emission wavelengths 488/515 nm was utilized in all experiments. The signal was amplified in the photomultiplier tube with a voltage biased at 600 V and the photocurrent was monitored with the rise time set to 0.5 s. The capillary was maintained at 25 °C and the separation voltage was +30 kV with an initial voltage rise 2.5 kV/s. During the separation, from the 35<sup>th</sup> minute an internal pressure of 25 mbar was applied to accelerate the separation. Vials containing BGE both for the capillary rinsing and for the analysis were replenished after each run using replenishment system of the Agilent G7100A CE System.

#### 2.5. Reagent solutions

The stock solution of 8 mM NDA was prepared in mixture of MeOH and IPA (4:1) dried over molecular sieves (3Å, Sigma-Aldrich), stored in a refrigerator in amber microtubes at 4 °C and used within a week. The stock solution of 250 mM NaCN was prepared in DI water, stored in refrigerator at 4 °C and used within a week. Stock solutions (20 mM) of individual AAs were prepared in DI water except for Tyr and Cyn, which were prepared in 0.1 M HCl and 0.1 M NaOH, respectively. Stock solution of the reaction buffer was prepared by dissolving the appropriate weight of H<sub>3</sub>BO<sub>3</sub> in 80 % of final volume of DI water, then the solution was adjusted to the pH 9.8 by addition of NaOH. Adjusted solution was quantitatively transferred to the volumetric flask and filled to the calibration mark with DI water giving the stock solution of 200 mM H<sub>3</sub>BO<sub>3</sub>/NaOH, pH 9.8. The stock solution of the reaction buffer was stored at laboratory temperature for a week and used for preparation of the working solutions of NDA, NaCN and model sample. The working concentration of the reaction buffer was always 100 mM H<sub>3</sub>BO<sub>3</sub>/NaOH, pH 9.8.

#### 2.6. Model sample

Aliquots of the stock solution of a mixture of 21 AAs were stored in refrigerator at -20 °C. Before



**Figure 2.** The electropherograms of AA-CBIs of the model sample using **TDLFP (A)** and **EMMA (B)** approach. The electropherograms are depicted in the same scale. **A:** Sequential injections: NDA, model sample, NaCN by pressure of 25 mbar for 6 s each zone; reaction time: 5 min. **B:** Sequential injections: NDA, model sample, NDA and NaCN by pressure of 50 mbar for 24, 7, 8 and 34 s, respectively; electrophoretic mixing -5 kV 65 s, reaction time: 3 min. **BGE:** 135 mM H<sub>3</sub>BO<sub>3</sub>/NaOH, 73 mM SDS, pH 9.00, 0.5 mM HP-β-CD, 6.7 % (v/v) 1P; **capillary:** 50/375 μm inner/outer diameters, 66/45 cm total/effective length; **voltage:** +30 kV; **temperature:** 25 °C; **detector:** ZETALIF™ LED 480, λ<sub>ex</sub> 480 nm, λ<sub>em</sub> 515 nm, photomultiplier tube voltage 600 V, rise time 0.5 s. **Peak numbering of AA CBIs:** Asn (1), Ser (2), Gln (3), Thr (4), His (5), Cynmono-CBI (6), Glu (7), Gly (8), Tyr (9), Ala (10), Asp (11), Tau (12), Ala-Gln (13), Val (14), Met (15), nVal (16), Trp (17), Ile (18), Leu (19), Phe (20), Arg (21), Cynbis-CBI (22) and Lys (23).

analysis, the aliquot was thawed at laboratory temperature and diluted in the volume ratio 1:1 in stock solution of the reaction buffer enriched with nVal (internal standard, IS) resulting in model sample composed of 5 μM of Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Ser, Tau, Thr, Trp, Tyr, Val, 15 μM Ala-Gln and 20 μM Cyn and Lys diluted in the reaction buffer (100 mM H<sub>3</sub>BO<sub>3</sub>/NaOH, pH 9.8) containing 2 μM nVal.

#### 2.7. EMMA-based method

The optimized derivatization conditions for EMMA comprises of sequential pressurized injection of 1 mM NDA in the reaction buffer with 12.5 % MeOH, sample in the reaction buffer and 1 mM NDA by pressure of 50 mbar for 24, 7 and 8 s, respectively. Finally, 2.5 mM NaCN in the reaction buffer was injected by pressure of 50 mbar for 34 s followed by electrophoretic mixing of the reactants by voltage of -5 kV for 65 s. The purpose of the hydrodynamic injection of the plug of NaCN was to keep the injected plugs in the capillary, which are pushed out by electroosmotic flow during the electrophoretic mixing. The reaction was performed for 3 minutes



at 25 °C. Total length of injected plugs was responsible for lowering the separation efficiency, thus pre-concentration by sweeping was applied. Sweeping plug comprised of 100 mM SDS dissolved in the stock solution of the reaction buffer (200 mM H<sub>3</sub>BO<sub>3</sub>/NaOH, pH 9.8) was injected prior the injection of the reactants by pressure of 50 mbar for 60 s. When the separation voltage (+30 kV) was applied, AA-CBIs interacting with SDS micelles were swept towards the capillary inlet. For detailed optimization see [5].

### 2.8. TDLFP-based method

TDLFP is the only diffusion driven mixing applicable for more than two reactants. The optimized derivatization conditions for TDLFP comprises of sequential pressurized injection of 1.5 mM NDA in the reaction buffer with 50 % MeOH, sample in the reaction buffer and 10 mM NaCN in the reaction buffer (each plug 25 mbar for 6 s). Laminar flow was induced by injection of BGE by pressure of 25 mbar for 30 s. The reaction was conducted for 5 minutes at 25 °C. For more details see [4].

## 3. Results and Discussion

### 3.1. Comparison of TDLFP- and EMMA-based methods

The presented EMMA and TDLFP mixing strategies for the on-capillary derivatization of AAs were at first compared in terms of sensitivity and repeatability. EMMA is characterized by improved limits of detection (S/N = 3) and quantification (S/N = 10) by a factor of 2–12. *Figure 2* shows the electropherograms of the model sample derivatized by TDLFP (*Figure 2A*) and EMMA (*Figure 2B*) under the optimal conditions. The electropherograms are depicted in the same scale. The peak heights in the electropherograms shows higher degree of conversion most likely originating in increased efficiency of the mixing in the case of EMMA. Intra- and inter-day repeatabilities were measured using the AAs model sample derivatized both by EMMA and TDLFP. Gradual decrease in peak areas was observed, which cannot be corrected using internal or external standardization. It was concluded to be due to cross-contamination leading to instability of the reagent solutions. To overcome this issue, it was sufficient to replenish the reagents

after 4 analyses. Comparable precision in terms of migration times was observed in the case of TDLFP and EMMA, but the EMMA-based method provided on average 2× better precision in peak areas without any correction applied. Supporting data are listed in Tables 1 and 2 in the article [5].

The competition of AAs for NDA/NaCN has been observed previously in on-capillary derivatization [4,6] and therefore both competition and matrix effects were studied. Calibration curves for a single representative analyte (Ile) were obtained by standard addition for different matrixes with varying content of compounds with a primary amine group (the model sample, commercial culture medium and human plasma). The slopes of the calibration curves for TDLFP varied in the range of -21.1-27.8 % with respect to the slope obtained for standard addition of Ile to the model sample. In the case of EMMA, the slopes of the calibration curves varied in the range of -3.0-3.3 %. The results indicate that competition among AAs is not significant when mixing of reactants by EMMA is used. It is probably due to the partial pre-separation of AAs in the course of electrophoretic mixing. Invariable slopes also indicate that the mixing of reactants by EMMA is not significantly affected by sample matrix effects.

## 4. Conclusion

Automatic on-capillary derivatization of AAs with NDA/NaCN was achieved by two fundamentally different mixing strategies, EMMA and TDLFP. The EMMA provided a higher mixing efficiency, lower susceptibility to competition effects and no sample matrix-related deterioration in comparison with TDLFP. Quantification precision was sufficient for the most of AAs when the reagent solutions were frequently replenished. The EMMA-based method showed potential for use on complex biological samples, such as human plasma, or culture media used in assisted reproduction to embryo cultivation.

## Acknowledgement

Financial support granted by the Czech Science Foundation (Projects No. P206/12/G014) is highly acknowledged.

## REFERENCES

- [1] Wu, G.Y.: Amino acids: metabolism, functions, and nutrition, *Amino Acids*, 2009 **37**, 1–17, <http://dx.doi.org/10.1007/s00726-009-0269-0>.
- [2] Glatz, Z.: On-capillary derivatisation as an approach to enhancing sensitivity in capillary electrophoresis, *Electrophoresis*, 2015 **36**, 744–763, <http://dx.doi.org/10.1002/elps.201400449>.
- [3] Demontigny, P.; Stobaugh, J.F.; Givens, R.S.; Carlson, R.G.; Srinivasachar, K.; Sternson, L.A.; Higuchi, T.: Naphthalene-2,3-dicarboxaldehyde/cyanide ion—a rationally designed fluorogenic reagent for primary amines, *Anal. Chem.*, 1987 **59**, 1096–1101, <http://dx.doi.org/10.1021/ac00135a007>.
- [4] Celá, A.; Mádr, A.; Dědová, T.; Pelcová, M.; Jeřeta, M.; Žáková, J.; Crha, I.; Glatz, Z.: MEKC-LIF method for analysis of amino acids after on-capillary derivatization by transverse diffusion of laminar flow profiles mixing of reactants for assessing developmental capacity of human embryos after in vitro fertilization, *Electrophoresis*, 2016 **37**, 2305–2312, <http://dx.doi.org/10.1002/elps.201500587>.
- [5] Celá, A.; Mádr, A.; Glatz, Z.: Electrophoretically mediated microanalysis for simultaneous on-capillary derivatization of standard amino acids followed by micellar electrokinetic capillary chromatography with laser-induced fluorescence detection, *J. Chromatogr. A*, 2017 **1499**, 203–210, <http://dx.doi.org/10.1016/j.chroma.2017.03.080>.
- [6] Weng, Q.F.; Jin, W.R.: Determination of free intracellular amino acids in single mouse peritoneal macrophages after naphthalene-2,3-dicarboxaldehyde derivatization by capillary zone electrophoresis with electrochemical detection, *Electrophoresis*, 2001 **22**, 2797–2803, <http://dx.doi.org/10.1002/>.

# MOLECULAR GLYCOHISTOPATHOLOGY BY CAPILLARY ELECTROPHORESIS

BOGLÁRKA DÖNCZÓ<sup>1,2</sup> AND ANDRÁS GUTTMAN,<sup>1,2\*</sup>

1 Horváth Csaba Laboratory of Bioseparation Sciences, Research Centre for Molecular Medicine, University of Debrecen, Nagyerdei krt. 98. Debrecen 4032 HUNGARY

2 MTA-PE Translational Glycomics Research Group, University of Pannonia, Egyetem u. 10, Veszprém 8200 HUNGARY

**Abstract** – N-linked glycans from standard glycoproteins, human serum and mouse tissue samples were investigated by capillary electrophoresis laser induced fluorescence (CE-LIF) detection. Intact, formalin treated and formalin-fixed, paraffin-embedded (FFPE) samples were used. FFPE samples were deparaffinized firstly, all of the samples solubilized with radioimmunoprecipitation assay (RIPA) buffer and digested with PNGase F endoglycanase enzyme for the N-glycan release. This step followed by fluorophore labeling and analysis by capillary electrophoresis. No significant differences were detected in the N-glycome profile at any investigated samples. The FFPE mouse tissue samples from lung have been used for N-glycan profiling. From this tissue sample, sixteen different N-linked carbohydrate structures were identified with exoglycosidase sequencing and GUcal software.

**Keywords:** FFPE, glycomics, capillary electrophoresis

## 1. Introduction

Since 1893, formalin was used for tissue fixation [1]. Furthermore, formalin fixation combined with paraffin embedment became the most prevalent method to preserve tissues from degradation [2]. This type of retained tissue is routinely prepared for pathological investigations, thus formalin fixed paraffin embedded (FFPE) tissue samples became almost exclusively used for long-term storage in histopathological laboratories and hospitals. A large number of archival tissue banks were established worldwide. FFPE specimens can serve as a valuable alternative for fresh frozen biopsy samples, which should be stored at -80°C [3].

The aim of our study was to develop a technique which could be suitable for global N-glycosylation profiling of FFPE tissues using high performance capillary electrophoresis analysis with laser induced fluorescence.

## 2. Experimental

### 2.1. Samples and Measurements

Fetuin, immunoglobulin G (IgG), ribonuclease B, human serum, radioimmunoprecipitation assay (RIPA) buffer, maltose, sodium cyanoborohydride, formaldehyde, paraffin and all other chemicals were from Sigma-Aldrich (St. Louis, MO). SCID male mice were used for the analyses. Animal-model

protocols were carried out in accordance with the Guidelines for Animal Experiments and were approved by the Institutional Ethics Committee at the National Institute of Oncology, Budapest, Hungary (permission number: 22.1/722/3/2010). PNGase F, *Arthrobacter ureafaciens* sialidase (ABS), Bovine kidney fucosidase (BKF), Jack bean galactosidase (JBG), Coffee bean  $\alpha$ -galactosidase (CBG) and Jack Bean hexosaminidase (JBH) were from ProZyme (Hayward, CA). The 8-aminopyrene-1,3,6-trisulfonate (APTS) and the maltooligosaccharide ladder were from SCIEX (Brea, CA).

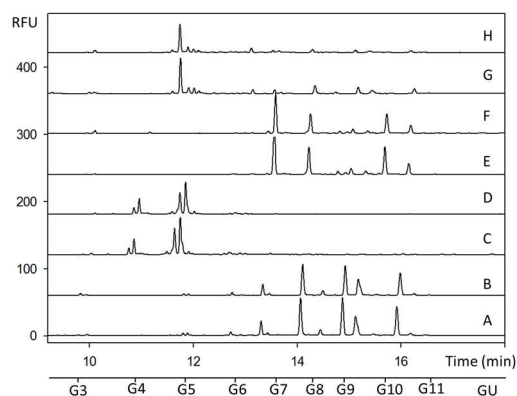
### 2.2. Theoretical Methodologies

Glucose Unit values of the peaks of interest are calculated by the GUcal software [4] ([www.gucal.hu](http://www.gucal.hu)) allowing adequate structural assignment using the built in database and the NIBRT based glycan database.

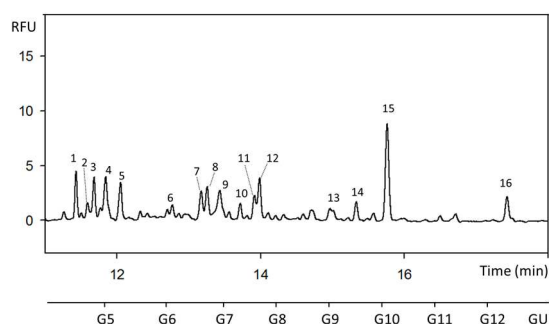
## 3. Results and Analysis

First the effect of formalin fixation and paraffin embedding were investigated on standard glycoproteins, human serum and mouse tumor tissue samples. Figure 1 represent the comparison of the APTS labeled N-glycan profiles of intact (A, C, E, G) and formalin-fixed, paraffin-embedded (B, D,

\*Correspondence: andras.guttman@hlbs.org



**Figure 1.** CE-LIF traces of APTS labeled released glycans from IgG (A, B), fetuin (C, D), ribonuclease B (E, F) and human serum (G, H). Trace A, C, E and G: not treated control; trace B, D, F and H: formalin-fixed paraffin-embedded. Separation conditions: NCHO coated capillary, 50 cm effective length (60 cm total), NCHO separation gel-buffer,  $E = 500$  V/cm, temperature  $25^{\circ}\text{C}$ , Injection: 1 psi/5 s. The lower X axis shows the corresponding GU values.



**Figure 2.** CE-LIF profile of APTS labeled released glycans from FFPE mouse lung tissue sample. Separation conditions were the same as in Figure 1.

F, H) IgG (trace A and B), fetuin (trace C and D), RNase B (trace E and F) and human serum (Trace G and H). Y axis represents the relative fluorescent units (RFU). The two X axes show the migration time and the GU values of the APTS labeled maltooligosaccharide ladder which was used as standard for the experiments. Furthermore, APTS labeled maltose was injected with every sample as an internal standard for the trace alignment. No apparent differences were observed between the corresponding capillary electrophoresis traces. This result suggests that formalin fixation and paraffin embedment have no effect on the N-glycosylation of the investigated samples [5].

**Table 1:** Exoglycosidase array reaction mixtures

Exoglycosidase enzyme	a	b	c	d	e	f
ABS	-	+	+	+	+	+
BKF	-	-	+	+	+	+
JBG	-	-	-	+	+	+
CBG	-	-	-	-	+	+
JBH	-	-	-	-	-	+

**Table 2.** APTS-labeled mouse lung N-glycans, sequenced by exoglycosidase array and identified by GUCal software.

Peak#	MT [min]	GU	Structure
1	11.429	4.549	A2G(4)2S(6,6)2
2	11.588	4.721	A2G(4)2S(3,6)2
3	11.679	4.819	F(6)A2G(4)2S(6,6)2
4	11.842	4.996	F(6)A2G(4)2S(3,6)2
5	12.05	5.240	A1[3]G(4)2S(3)1
6	12.771	6.096	A2G(4)2S(6)1
7	13.171	6.607	F(6)A2[6]G(4)2S(6)1
8	13.254	6.713	M5
9	13.429	6.936	F(6)A2G1Ga1S1
10	13.727	7.326	F(6)A2[3]G(4)2S(6)1
11	13.913	7.595	M6
12	13.983	7.691	F(6)A2[3]G(4)2S(3)1
13	14.967	9.039	A2G(4)2
14	15.333	9.530	M8
15	15.762	10.106	F(6)A2G(4)2
16	17.433	12.366	FA2G2Ga2

Mouse lung tissue was used for the analysis, both fixed with formalin and embedded in paraffin. After the deparaffinization and the solubilization, the sample was released enzymatically, labeled with a charged fluorophore then analyzed by CE (Figure 2). The N-glycan pool of the lung tissue contains

mostly sialylated structures (GU=4-8) and some neutral (GU=9-13) oligosaccharides.

N-glycans from lung FFPE tissue were identified by their GU values during exoglycosidase array based carbohydrate sequencing. The reaction mixtures of the array contained *Arthrobacter ureafaciens* sialidase which remove  $\alpha(2-3,6,8)$  linked sialic acids; Bovine kidney fucosidase which release  $\alpha(1-2,3,4,6)$  fucoses; Jack bean galactosidase which remove  $\beta(1-4,6)$  linked galactoses; Coffee bean  $\alpha$ -galactosidase which cut all  $\alpha(1-3,4,6)$  galactose residues and Jack Bean hexosaminidase which remove the  $\beta(1-2,4,6)$  linked N-acetylglucosamines. 0.5 U was used from each exoglycosidase enzyme. The fluorophore labeled samples were digested at the same time. The digestion was incubated at 37°C overnight in 50 mM ammonium-acetate buffer (pH 5.5). The combinations of the reaction mixtures (a-f) are shown in Table 1. The ammonium-acetate content was removed by centrifugal vacuum evaporator drying.

Table 2 shows the glycan structures of the N-glycome of FFPE lung tissue which appeared in Figure 2. The calculation of the GU values was made by GUCal software. For the structural identification, publicly available databases were used as it was mentioned above [6].

#### 4. Discussion

All N-linked glycans of standard glycoproteins, human serum and mouse tissue samples were liberated by PNGaseF digestion before and after the fixation and embedding processes. The released glycans were labeled with fluorescent dye (APTS) and analyzed by capillary electrophoresis - laser induced fluorescent detection.

#### 5. Conclusion

Our study revealed that N-glycan profiles were identical before and after the formalin fixation and the paraffin embedding. It was demonstrated that FFPE samples could be useful for the identification of glycan structures which is the first step on the path to utilize huge sample collections located in hospitals for prospective and retrospective biomedical and biopharmaceutical studies.

#### Acknowledgement

The research was supported by the MTA-PE Translational Glycomics program (#97101), the NKFIH K116263 grant and the BIONANO\_GINOP- 2.3.2-15-2016-00017 project.

#### REFERENCES

- [1] Blum, F.: Formaldehyde, *Enzyklopadie d mikroskop Technik, Urban & Schwarzenberg* 1910 **1**(1): 478-92.
- [2] Fox, C.H.; Johnson, F.B.; Whiting, J.; Roller, P.P.: Formaldehyde fixation, *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* 1985; **33**(8):845-53.
- [3] Magdeldin, S.; Yamamoto, T.: Toward deciphering proteomes of formalin-fixed paraffin-embedded (FFPE) tissues, *Proteomics* 2012; **12**(7):1045-58.
- [4] Jarvas, G.; Szigeti, M.; Guttman, A.: Structural identification of N-linked carbohydrates using the GUCal application: A tutorial, *Journal of proteomics* 2017.
- [5] Donczo, B.; Szigeti, M.; Ostoros, G.; Gacs, A.; Tovari, J.; Guttman, A.: N-Glycosylation analysis of formalin fixed paraffin embedded samples by capillary electrophoresis, *Electrophoresis* 2015 **37** (17-18): 2292-6.
- [6] Donczo, B.; Szarka, M.; Tovari, J.; Ostoros, G.; Csanky, E.; Guttman, A.: Molecular glycopathology by capillary electrophoresis: Analysis of the N-glycome of formalin-fixed paraffin-embedded mouse tissue samples, *Electrophoresis* 2017; **38**(12):1602-8.

# SEMIQUANTITATIVE ASSESSMENT OF DISHEVELLED 3 PHOSPHORYLATION STATUS BY MASS SPECTROMETRY

KATEŘINA HANÁKOVÁ,<sup>1,2</sup> DAVID POTĚŠIL,<sup>2</sup> ONDŘEJ BERNATÍK,<sup>3</sup> IGOR ČERVENKA,<sup>3</sup> MATĚJ RÁDSETOULAL,<sup>3</sup> VÍTĚZSLAV BRYJA,<sup>3</sup> ZBYNĚK ZDRÁHAL<sup>1,2\*</sup>

1 National Centre for Biomolecular Research, Faculty of Science, Masaryk University, Brno, 62500, Czech Republic

2 Research Group Proteomics, Central European Institute of Technology, Masaryk University, Brno, 62500, Czech Republic

3 Department of Experimental Biology, Faculty of Science, Masaryk University, Brno, 62500, Czech Republic

**Abstract** – We focused on human Dishevelled 3 protein (hDVL3), an essential component of Wnt signalling pathway that contributes to their regulation. We performed mass spectrometry-based analysis of hDVL3 phosphorylations induced by eight associated kinases, which revealed several dozens of phosphorylation sites. Main outcome of this study was the description of DVL phosphorylation “patterns” induced by individual kinases.

**Keywords:** phosphorylation, dishevelled 3, mass spectrometry, CK1 $\epsilon$ , NEK2

## 1. Introduction

Reversible protein phosphorylation belongs to posttranslational modification with high biological significance mainly due to its role in regulation cellular processes such as gene expression, cell division, signal transduction, metabolism, differentiation, apoptosis etc. [1].

Dishevelled-3 (DVL3) is a protein involved in the Wnt signalling pathways and gets heavily phosphorylated in response to pathway activation by Wnt ligands [2].

In our study, we combined SDS-PAGE separation of hDVL3 immunoprecipitates with titanium dioxide phosphopeptide enrichment followed by LC-MS/MS. Data were processed using Skyline software to obtain a semiquantitative assessment of occupancy of phosphorylation sites induced by individual kinases.

## 2. Experimental

### 2.1. Cell culture and transfection

HEK293 cell line was used for this study. Cells were seeded in 15 cm dishes and transfected at a confluence of 60%. Two days after transfection, after reaching confluence, they were harvested for immunoprecipitation. Chilled NP40 lysis buffer with protease inhibitors, DTT, phosphatase

inhibitors and NEM was used for cell lysis. Lysate was incubated with antibody Anti FLAG M2.

### 2.2. Gel electrophoresis, protein digestion and phosphopeptide enrichment

Immunoprecipitates were separated on a SDS-PAGE gel electrophoresis, separated, fixed with acetic acid in methanol, and stained with Coomassie Brilliant Blue for 1 hour and partially destained.

Corresponding 1-D bands were excised. After destaining, the proteins in gel pieces were incubated with 10mM DTT at 56 °C for 45 min. After removal of DTT excess samples were incubated with 55mM IAA at room temperature in darkness for 30 min, then alkylation solution was removed and gel pieces were hydrated for 45 min at 4 °C in digestion solution (5 ng/ $\mu$ l trypsin, sequencing grade, Promega, Fitchburg, Wisconsin, USA, in 25mM AB). The trypsin digestion proceeded for 2 hours at 37 °C on Thermomixer (750 rpm; Eppendorf, Hamburg, Germany). Subsequently, the tryptic digests were subsequently cleaved by chymotrypsin (5 ng/ $\mu$ l, sequencing grade, Roche, Basel, Switzerland, in 25mM AB) for 2 hours at 37 °C. Digested peptides were extracted from gels using 50% ACN solution with 2.5% formic acid and concentrated in speedVac concentrator (Eppendorf, Hamburg, Germany). The aliquot (1/10) of sample was transferred to LC-MS vial and concentrated under vacuum. Water was used to get 15  $\mu$ l of

\*E-mail: [zdrahal@sci.muni.cz](mailto:zdrahal@sci.muni.cz) Phone: +420-54949-8258

MGETKIIYHLDGQETPYLVKLLPLPAERVTLADFKGVLQRP	SYKFFFKSMD	50
DDFGVVKEEISDDNAKLPCFNRRVSWLVSAEGSHDPAPFCADNPSELP		100
PPMERGGIGDSRPPSFHPHAGGGSQENLDNDTEIDSLVSAQRERPRRRD		150
GPEHATRLNGTAKGERRREPGGYDSSSTLMSSSELETTFFFDSEDDSTSR		200
FSSSTEQSSASRLMRRHKRRRRKQKVSRIERSSSSFSSITDSTMSLNIITV		250
ILNMEKYNFLGISIVGQSNERGDGGIYIGSIMKGGAVTADGRIEFGDMLL		300
QVNEINFENMSNDDAVRVLREIVHKPGPITLTVAKCWDSPRGCFILPRS		350
EP IRPIDPAAVVSHTAAMTGTFFPAYGMSPSLSTITSTSSSITSSIPDTER		400
LDDFHLSTHSDMAAIVKAMASPESGLEVRDRMWLKITIPNAFIGSDVVDW		450
LYHNVEGFTDRREARKYASNLLKAGFIRHTVNKIIFSEQCYIIFGDLCGN		500
MANLSLHDHDGSSGASDQDTLAPLPHPGAAPWPMAPFYQYPPPPHPYNPH		550
PGFPPELGYSYGGGSASSHSEGRSSGSNRSRSGDRRKEKDPKAGDSKSGG		600
SGSES DHTTRSSLRGPRERAPSERSGPAASEHSHRSHHSLASSLRSHHTH		650
PSYGGPPGVPPLYGPPMLMPPPPAAMGPPGAPPGRDLASVPPELTASRQS		700
FRMAMGNPSEFFVDVM		

**Figure 1. Comparison of phosphorylation sites of DVL3 induced by CK1ε and NEK2.** Experimentally determined phosphorylation sites by CK1ε and NEK2 are indicated by green background color. Phosphorylation sites identified only by NEK2 are indicated by red background color.

peptide solution and directly analyzed by LC-MS/MS for protein identification (ID run).

The rest of peptide mixture was used for phosphopeptide analysis. MS PhosphoMix 1, 2, 3 Light (Sigma Aldrich) were added to the samples before phosphopeptide enrichment step in concentration 0.1pmol. Phosphopeptides were enriched using Pierce Magnetic Titanium Dioxide Phosphopeptide Enrichment Kit (Thermo Scientific, Waltham, Massachusetts, USA) according to manufacturer protocol and eluted into LC-MS vial. Solution was concentrated under vacuum to volume below 5 µl, dissolved in water and 0.6 µl of 5% FA to get 15 µl of peptide solution before LC-MS/MS analysis.

### 2.3. Mass spectrometry

LC-MS/MS analyses of peptide mixture were done using RSLCnano system connected to Orbitrap Elite hybrid spectrometer (Thermo Fisher Scientific) with ABIRD (Active Background Ion Reduction Device; ESI Source Solutions) and Digital PicoView 550 (New Objective) ion source (tip rinsing by 50% acetonitrile with 0.1% formic acid) installed. Prior to LC separation, tryptic digests were online concentrated and desalted using trapping column (100 µm × 30 mm) filled with 3.5-µm X-Bridge BEH 130 C18 sorbent (Waters). After washing of trapping column with 0.1% FA, the peptides were eluted (flow 300 nl/min) from the trapping column onto Acclaim Pepmap100 C18 column (3 µm particles, 75 µm × 500 mm; Thermo Fisher Scientific) by 65 min long gradient. Mobile

phase A (0.1% FA in water) and mobile phase B (0.1% FA in 80% acetonitrile) were used in both cases. The gradient elution started at 1% of mobile phase B and increased from 1% to 56% during the first 50 min (30% in the 35th and 56% in 50th min), then increased linearly to 80% of mobile phase B in the next 5 min and remained at this state for the next 10 min. Equilibration of the trapping column and the column was done prior to sample injection to sample loop. The analytical column outlet was directly connected to the Digital PicoView 550 ion source.

MS data were acquired in a data-dependent strategy selecting up to top 6 precursors based on precursor abundance in the survey scan (350-2000 m/z). The resolution of the survey scan was 60 000 (400 m/z) with a target value of 1×10<sup>6</sup> ions, one microscan and maximum injection time of 200 ms. High resolution (resolution 15 000 at 400 m/z) HCD MS/MS spectra were acquired with a target value of 50 000. Normalized collision energy was 32 % for HCD spectra. The maximum injection time for MS/MS was 500 ms. Dynamic exclusion was enabled for 45 s after one MS/MS spectra acquisition and early expiration was disabled. The isolation window for MS/MS fragmentation was set to 2 m/z.

The analysis of the mass spectrometric RAW data files was carried out using the Proteome Discoverer software (Thermo Fisher Scientific; version 1.4) with in-house Mascot (Matrixscience; version 2.4.1) search engine utilization. MS/MS ion searches were done against in-house database

containing expected protein of interest with additional sequences from cRAP database (downloaded from <http://www.thegpm.org/crap/>). Mass tolerance for peptides and MS/MS fragments were 7 ppm and 0.03 Da, respectively. Oxidation of methionine, deamidation (N, Q) and phosphorylation (S, T, Y) as optional modification, carbamidomethylation of C as fixed modification and three enzyme miss cleavages were set for all searches. The phosphoRS feature was used for phosphorylation localization.

Quantitative information was assessed and manually validated in Skyline software (Skyline daily 3.1.1.8884).

### 3. Results and Analysis

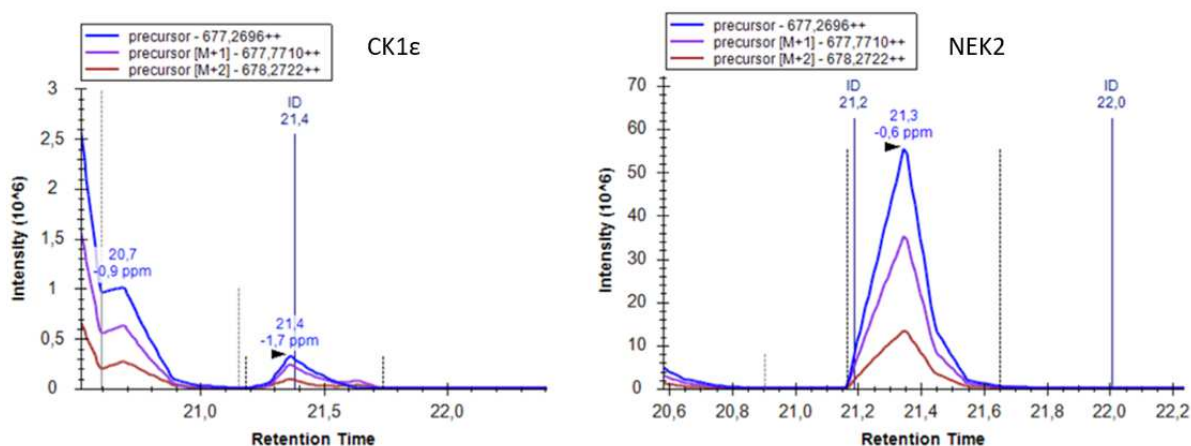
#### 3.1. Identification of phosphorylation sites

Phosphorylation is important for protein function and regulation. We analysed phosphorylation status of human DVL3 induced by eight individual Ser/Thr kinases that were previously reported or identified by unbiased MS screen for DVL associated kinases. DVL3 contains 131 serines/threonines, which can be potentially phosphorylated. In total, we managed to identify 88 Ser/Thr phosphorylation sites and one tyrosine phosphorylation site in DVL3.

#### 3.2. Phosphorylations induced by CK1 $\epsilon$ and NEK2

Based on our experiment, we created a phosphorylation “map” of DVL protein that described complex phosphorylation “fingerprint” for each tested kinase. Eight of the kinases used to induced phosphorylation include CK1 $\epsilon$  and NEK2. Figure 1. shows a qualitative comparison of the identified phosphorylation sites using these two kinases. In the case of CK1 $\epsilon$  induction, we identified 77 phosphorylation sites, and in the case of NEK2, we determined 87 phosphorylation sites from a total of 131 possible Ser/Thr phosphorylation sites in the DVL3 protein.

Next to qualitative characterization, we performed semiquantitative comparison of occupancy of phosphorylation sites induced by individual kinases. The Skyline software was used for this evaluation. We compared the individual phosphorylated peptides based on the peak area. Figure 2. shows a comparison of the selected peptide phosphorylated in position of S204 by CK1 $\epsilon$  and NEK2. Peak area was determined for CK1 7.60e6 and for NEK2 1.07e9. Subsequently, double normalization of the data was performed using the set of phosphopeptide standards (added to the sample prior phosphoenrichment step) and by unphosphorylated peptides identified in ID run. The resulting areas (CK1: 1.23e7 and NEK2: 1.10e9) were compared with each other.



**Figure 3. Semiquantitative analysis of phosphorylation site S204 on peptide FSSpSTEQSSASR induced by CK1 $\epsilon$  and NEK2.** Precursor and selected fragment traces of corresponding hDVL3 phosphopeptide are shown for CK1 $\epsilon$  and NEK2 (in Skyline). The highest signal intensity was reached in the case of NEK2.



## 4. Discussion

In our study, we focus on determination of phosphorylation sites of DVL3 by MS induced by eight kinases. In this study was determined 88 Ser/Thr phosphorylations from 131 sites, which can be potentially phosphorylated and 1 tyrosine phosphorylation.

We identified CK1 $\epsilon$ -induced phosphorylation on 77 unique sites and 10 more phosphorylation sites induced by NEK2. Previous studies in various experimental systems identified several phosphorylation sites spread throughout the structure of Dvl protein [3, 4]. Our data clearly demonstrate that phosphorylation of Dvl protein is extensive and the number of phosphorylated sites exceeds 60.

## 5. Conclusion

An approach based on SDS-PAGE separation of DVL3 immunoprecipitates, TiO<sub>2</sub> phosphoenrichment followed by LC-MS/MS analysis and data processing in Skyline SW was utilized for evaluation of semiquantitative differences in phosphorylation level of hDVL3 particular sites within the set of eight selected kinases.

We observed differences in phosphorylation profiles induced by individual kinases, as indicated in Figure 1. Based on our results, a “comprehensive map” of phosphorylations of human DVL3 will be created.

## Acknowledgement

*This work was carried out with the support of project CEITEC 2020 (LQ1601), with financial support from the Ministry of Education, Youth and Sports of the Czech Republic under the National Sustainability Programme II. CIISB research infrastructure project LM2015043 funded by MEYS CR is gratefully acknowledged for financial supporting the LC-MS/MS measurements at the Proteomics Core Facility. We thank for the support by Czech Science Foundation project (no. 15-21789S).*

## REFERENCES

- [1] Kersten, B., Agrawal, G. K., Iwahashi, H., Rakwal, R.: Plant phosphoproteomics: A long road ahead, *Proteomics*, 2006 **6** (20), 5517–5528
- [2] Bernatík, O., Šedová, K., Schille, C., Ganji, S. R., Červenka, I., Trantírek, L., Schambony, A., Zdráhal, Z., Bryja, V.: Functional analysis of Dishevelled-3 phosphorylation identifies distinct mechanisms driven by casein kinase 1 epsilon and frizzled5. *Journal of Biological Chemistry*, 2014 **34** (289), 23520-23533 doi:10.1074/jbc.M114.590638
- [3] Yanfeng, W. A., Berhane, H., Mola, M., Singh, J., Jenny, A., Mlodzik, M.: Functional dissection of phosphorylation of Dishevelled in *Drosophila*, *Dev. Biol.*, 2011 **360**, 132–142
- [4] Klimowski, L. K., Garcia, B. A., Shabanowitz, J., Hunt, D. F., Virshup, D. M.: Site-specific casein kinase 1 $\epsilon$ -dependent phosphorylation of Dishevelled modulates  $\beta$ -catenin signaling. *FEBS J.*, 2006 **273**, 4594–4602

# MICROCHIP ELECTROPHORESIS WITH CONDUCTIVITY DETECTION IN THE ANALYSIS OF PHARMACEUTICAL AND BIOLOGICAL SAMPLES

JASNA HRADSKI,\* RÓBERT BODOR, AND MARIÁN MASÁR

Department of Analytical Chemistry, Faculty of Natural Sciences, Comenius University in Bratislava, Ilkovičova 6, Mlynská dolina, Bratislava, SK-84215, SLOVAKIA

**Abstract** – Microchip electrophoresis has so far been coupled to various detection techniques in order to achieve selective and also sensitive detection. Among them conductivity detection used in this study offers universal response to all studied analytes, relatively low price per analysis as well as easy miniaturization of all components of the detector. In this study the use of contact conductivity detection integrated directly on the microchip is shown. Even though conductivity detection is characterized by lower sensitivity than some of the other detection techniques used, it can be applied to the determination of analytes in wide range of samples, e.g., biological, pharmaceutical, environmental, food or forensic samples. Here, pharmaceutical, i.e., buserelin acetate, and biological, cerebrospinal fluid, samples have been analyzed using two different electrophoretic techniques, zone electrophoresis and isotachopheresis. Acetate as a pharmaceutical counterion has been determined in buserelin acetate, whereas inorganic cations (ammonium, calcium, magnesium, sodium and potassium) and anions (chloride, sulfate, nitrite and nitrate), whose change in the concentration can be an indicator of serious neurological diseases have been determined in cerebrospinal fluid of patients with suspected neurological diseases. Highly precise and reproducible determination of acetate was achieved using isotachopheresis. The use of microchip with coupled separation channels and column switching technique enabled the determination of both micro- and macroconstituents in cerebrospinal fluid.

**Keywords:** microchip electrophoresis, conductivity detection, pharmaceuticals, biological samples

## 1. Introduction

Microchip electrophoresis (MCE) is a miniaturized version of capillary electrophoresis. Main advantages of MCE in comparison to capillary electrophoresis include faster analysis, lower consumption of sample and reagents and thus lower production of waste [1].

One of the main differences between these two techniques is detection technique used. In conventional capillary electrophoresis UV/Vis detection is the most widely used detection technique, however given the extremely narrow channels, i.e., short optical pathway this technique did not find wide use in MCE [2]. Laser induced fluorescence characterized by high sensitivity and fast response was the first optical detection technique coupled to MCE [3]. Even nowadays this technique is the most often used detection technique with MCE. Main drawbacks of this technique are necessity of derivatization step for compounds that are not naturally fluorescent, which can be time consuming, large dimensions and limited ability for miniaturization of the instrumentation as well as its relatively high price [4].

Conductivity detection used in this work is considered universal detection technique for MCE. In comparison to fluorescence detection the use of fluorophore is eliminated. Also it can be implemented directly on the microchip and miniaturization of the conductivity instrumentation has no influence on the sensitivity of the response [5]. Despite its universal applicability main drawback of conductivity detection in comparison to laser induced fluorescence is relatively lower sensitivity.

Nevertheless, in this study applicability of conductivity detection to the MCE separations is shown through the determination of pharmaceutical counterion in peptide drug [6] and various inorganic ions in cerebrospinal fluid [7].

Drugs are often produced in a salt form to increase their stability and solubility. Precise determination of pharmaceutical counterions is necessary to confirm production of the correct salt form [8]. Acetate present in a cancer treatment drug buserelin as a pharmaceutical counterion was studied.

Analysis of cerebrospinal fluid is mainly used in diagnostics of neurological diseases. Determination of inorganic ions in cerebrospinal fluid is important

---

\*Correspondence: [hradski1@uniba.sk](mailto:hradski1@uniba.sk)

because change in the concentration of certain inorganic cations or anions can be an indicator of various serious diseases, Alzheimer's disease, multiple sclerosis or vascular dementia [9-11]. Inorganic ions commonly found in the cerebrospinal fluid, i.e., cations (ammonium, calcium, magnesium, sodium and potassium) and anions (chloride, sulfate, nitrite and nitrate) were chosen as suitable analytes.

In addition, MCE with conductivity detection has also been successfully applied to analysis of food, environmental, pharmaceutical and biological samples [12,13].

In order to achieve separation and subsequent quantification of studied analytes two of the most widely used electrophoretic techniques, zone electrophoresis (ZE) and isotachopheresis (ITP), were used. ZE is the simplest and most frequently used electrophoretic technique. Separation is carried out in one electrolyte, so called background electrolyte (BGE) and sample constituents are separated into zones based on their different migration velocities. ITP separation is based on the electric field gradient from leading (LE) to terminating (TE) electrolyte. This causes migration of all sample constituents with same velocity and zones of constituents are created between the zone of leading and terminating ions. Under isotachopheretic conditions analyte is present only in its own zone at constant concentration.

Microchip with coupled separation channels used for MCE separations in this study enables the use of so called column switching technique. That is a great benefit, when analytes having similar electromigration properties and being present in the sample at different concentration levels can be studied in one run [14].

## 2. Experimental

MCE separations were carried out on a poly(methylmethacrylate) microchip with coupled separation channels and integrated conductivity sensors (IonChip™ 3.0; Merck, Darmstadt, Germany). A schematic arrangement of the channels on the microchip as well as their filling with corresponding electrolytes for both ITP and ZE is shown in Fig.1. A design concept of MCE instrument is described in details elsewhere [14].

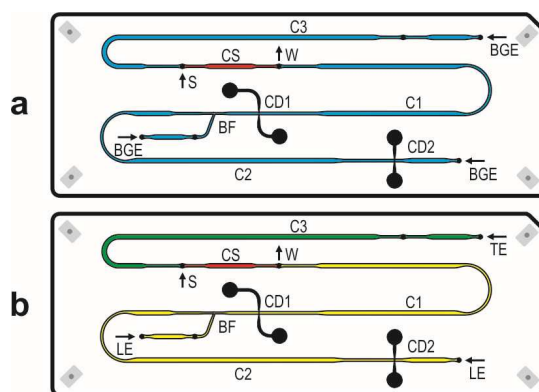


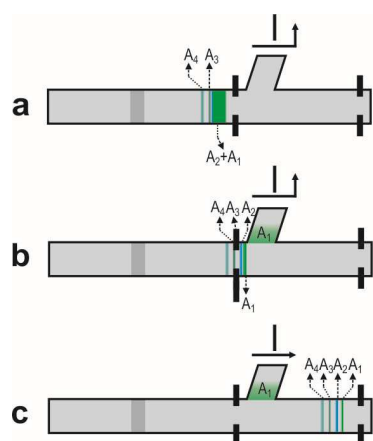
Figure 1. Microchip with coupled channels and integrated conductivity sensors. Electrolyte layout for (a) ZE, and (b) ITP separations, respectively. C1, C2 the first and the second separation channels, respectively; C3 non-separative channel; CS sample injection channel; CD1, CD2 conductivity sensors in the C1 and C2, respectively; BGE, LE, TE, S inlets of the microchip channels for BGE, LE, TE and sample solutions, respectively; W an outlet from the channels; BF bifurcation section of the coupled channels.

Electrolyte and electronic unit are the main operational components of MCE instrument. Electrolyte unit consists of peristaltic micropumps and membrane driving electrodes. Peristaltic micropumps are used for rinsing of the microchip channels and transport of electrolyte and sample solutions to the microchip. Another function of peristaltic micropumps is to suppress hydrodynamic flow by closing the inlets of the microchip. The membrane driving electrodes placed outside of the microchip suppress disturbances of the separation due to the bubble formation during the run [15].

Electronic unit delivers stabilized driving current to the counter-electrodes placed at the end of each separation channel, drives the peristaltic micropumps and interfaces the MCE instrument to a PC. This unit also includes the measuring electronics of the contact conductivity detectors. Monitoring of the analysis, automatic preparation of the run as well as collection of the data from conductivity detectors and their evaluation were done using MicroCE Win software, version 2.4 (Merck).

### 2.1. Preparation of Electrolytes and Samples

For the preparation of electrolyte and model sample solutions chemicals of p.a. purity were used (Sigma-Aldrich, Steinheim, Germany). Water demineralized by a Pro-PS water purification system (Labconco, Kansas City, KS, USA) and subsequently highly deionized by a circulation in a



**Figure 2.** Working steps of the ZE-ZE separation: (a) separation in the first separation channel; (b) continuation of the run with removal of the macroconstituents after detection in the first channel; (c) separation and detection of the transferred microconstituents in the second separation channel by switching direction of the driving current.  $A_{1-4}$  analytes,  $i$  driving current.

Simplicity deionization unit (Millipore, Molsheim, France) was used for preparation of the electrolyte and sample solutions.

For ITP analysis model and real samples were prepared in a 10% (v/v) TE. Pharmaceutical sample prepared from busserelin acetate (Merck) was diluted approximately 10-20 times prior to analysis.

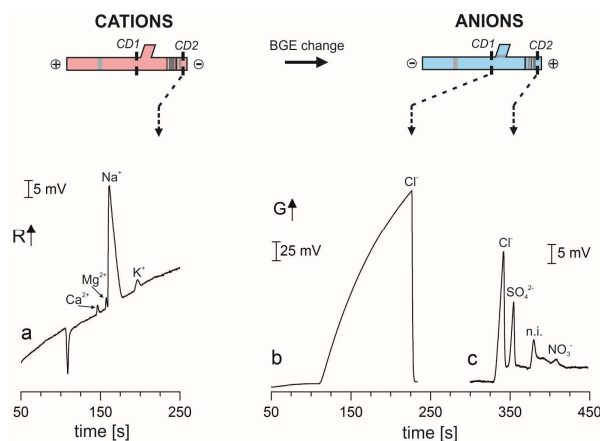
Cerebrospinal fluid samples were collected from three patients with symptoms of neurodegenerative diseases in the First Neurological Clinic on Faculty of Medicine, Comenius University in Bratislava and stored at  $-40\text{ }^{\circ}\text{C}$ . Prior to the analysis samples were defrosted and homogenized.

### 3. Results and Discussion

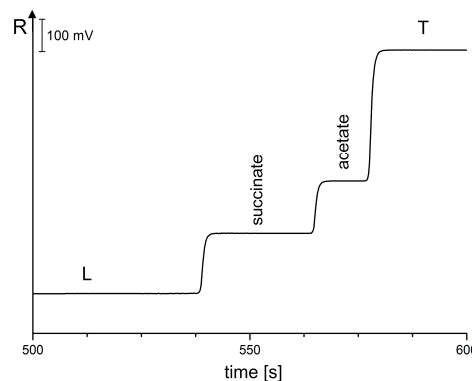
#### 3.1. ZE Separation of Inorganic Ions in Cerebrospinal Fluid

ZE separations of cations and anions were performed in two different propionate BGEs on the same microchip. Repeatability of migration time and peak area was evaluated from four repeated ZE runs of model samples at three different concentration levels. RSD of migration times were within 1.1% and RSD of peak area were up to 6.0% for both cations and anions.

Calibration graphs were constructed using the peak area as a function of concentration. For estimation



**Figure 3.** ZE separations of inorganic cations and anions in cerebrospinal fluid sample. (a) ZE separation of cations in 1500 times diluted cerebrospinal fluid sample in the second separation channel; (b, c) ZE-ZE separation of anions in 20 times diluted cerebrospinal fluid sample in the first and the second separation channel, respectively. Driving current was stabilized in both channels at  $50\text{ }\mu\text{A}$  for the separation of cations and  $30\text{ }\mu\text{A}$  for the separation of anions. *n.i.* not identified constituent,  $R$  resistance,  $G$  conductivity.



**Figure 4.** ITP separation of acetate in busserelin acetate sample. Loaded sample contained  $885\text{ mg L}^{-1}$  busserelin acetate and  $100\text{ mg L}^{-1}$  succinate.  $L$  leading ion,  $T$  terminating ion,  $R$  resistance.

of limit of detection (LOD) standard procedure based on three time signal to noise ratio ( $3 \times S/N$ ) was used. LODs were in the range  $0.003\text{--}0.012\text{ mg L}^{-1}$  for cations and  $0.019\text{--}0.067\text{ mg L}^{-1}$  for anions.

ZE was used for the determination of cationic constituents while due to relatively high chloride content ZE-ZE approach was employed for the analysis of anions (Fig.2). In this case chloride, which migrated first, was determined in the first separation channel and then removed from the separation pathway through bifurcation section. After switching the direction of the driving current

the rest of chloride with other anionic microconstituents were separated in the second separation channel and determined by conductivity sensors in the second separation channel (*Fig.2*).

Cerebrospinal fluid samples were analyzed after appropriate dilution. Each sample was analyzed for both cationic and anionic content. Repeatability of both migration time and peak area of the analytes in the cerebrospinal fluid samples were similar to those in model samples. Recovery of both cations and anions was in the range 90–106%. Electropherograms obtained from cationic and anionic analyses of cerebrospinal fluid are shown in *Fig.3*. Ammonium and nitrite were present in all analyzed samples under their LOD, while concentrations of other ions were in a good agreement with the data found in the literature. Small deviations can be ascribed to suspected diseases.

### 3.2. ITP Separation of Acetate in Buserelin Acetate

ITP separations were carried out in chloride/caproate electrolyte system at pH about 6.0. Determination of acetate was performed using two quantification methods: external calibration and internal standard method, with succinate used as an internal standard. Using external calibration method RSD of the acetate zone lengths ranged from 3 to 4% independently on the microchip or MCE instrument. The use of internal standard method led to much lower RSD (0.1–0.7%).

Two identical MCE instruments and two MCE microchips were used for studying of the long-term validity of ITP quantitative parameters. From the results obtained a very good possibility for chip-to-chip or device-to-device transfer of the ITP method was evident.

Three samples of buserelin acetate with different additions of acetate were used to evaluate recovery. Recovery was in the range 98–101%, which indicates a very good accuracy of the ITP method developed. Isotachopherogram obtained from the determination of acetate in buserelin acetate sample is shown in *Fig.4*.

## 4. Conclusion

MCE with contact conductivity detection has proven suitable for analysis of small inorganic and organic ions in pharmaceutical and biological samples. Determination of inorganic cations and anions in cerebrospinal fluid samples was performed on the same microchip in two relatively similar BGEs in 15 min. Most of the studied ions were successfully determined using contact conductivity detection. It was also shown that ITP performed on the same microchip is able to provide highly precise and accurate determination of major constituents in pharmaceuticals. Smooth chip-to-chip transfer of the analytical method indicates the long-term validity of the analytical results.

## Acknowledgement

The research was supported by the projects: APVV-0259-12, VEGA 1/0340/15 and UK/312/2017.

## REFERENCES

- [5] Hendrickx, S.; De Malsche, W.; Cabooter, D. An overview of the use of microchips in electrophoretic separation techniques: Fabrication, separation modes, sample preparation opportunities, and on-chip detection. In: Van Schepdael, A. (ed.). *Microchip capillary electrophoresis protocols* (Humana Press Inc., Totowa, New Jersey, USA) 2015, 3-17 DOI 10.1007/978-1-4939-2353-3\_1
- [6] Henry, C. S. (ed.) *Microchip capillary electrophoresis: methods and protocols* (Humana Press Inc., Totowa, New Jersey, USA) 2006 DOI 10.1385/1597450766
- [7] Harrison, D.J.; Manz, A.; Fan, Z.; Lüdi, H.; Widmer, H.M.: Capillary electrophoresis and sample injection systems integrated on a planar glass chip, *Anal. Chem.*, 1992 **64**(17), 1926–1932 DOI 10.1021/ac00041a030
- [8] Johnson, M.E.; Landers, J.P.: Fundamentals and practice for ultrasensitive laser-induced fluorescence detection in microanalytical systems, *Electrophoresis*, 2004 **25**(21-22), 3513–3527 DOI 10.1002/elps.200406086
- [9] Guijt, R.M.; Evenhuis, C.J.; Macka, M.; Haddad, P.R.: Conductivity detection for conventional and miniaturised capillary electrophoresis systems, *Electrophoresis*, 2004 **25**(23-24), 4032–4057 DOI 10.1002/elps.200406156

- [10] Hradski, J.; Drusková Chorváthová, M.; Bodor, R.; Sabo, M.; Matejčík, Š.; Masár M.: Quantitative aspects of microchip isotachopheresis for high precision determination of main components in pharmaceuticals, *Anal. Bioanal. Chem.*, 2016 **408**(30), 8669–8679 DOI 10.1007/s00216-016-9815-2
- [11] Hradski, J.; Bodor, R.; Masár M.: Sequential determination of inorganic cations and anions in cerebrospinal fluid by microchip electrophoresis, *Chromatographia*, 2014 **77**(21-22), 1461–1468 DOI 10.1007/s10337-014-2711-z
- [12] Štěpánová, S.; Kašička, V.: Determination of impurities and counterions of pharmaceuticals by capillary electromigration methods, *J. Sep. Sci.*, 2014 **37**(15), 2039–2055 DOI 10.1002/jssc.201400266
- [13] Basun, H.; Forssell, L.; Wetterberg, L.; Winblad, B.: Metals and trace elements in plasma and cerebrospinal fluid in normal aging and Alzheimer's disease, *J. Neural. Transm.-Park.*, 1991 **3**(4), 231–258 DOI 10.1016/0197-4580(92)90506-s
- [14] Lampl, Y.; Geva, D.; Gilad, R.; Eshel, Y.; Ronen, L.; Sarova-Pinhas, I.: Cerebrospinal fluid magnesium level as a prognostic factor in ischaemic stroke, *J. Neurol.*, 1998 **245**(9), 584–588 DOI 10.1007/s004150050249
- [15] Tohgi, H.; Abe, T.; Yamazaki, K.; Murata, T.; Isobe, C.; Ishizaki, E.: The cerebrospinal fluid oxidized NO metabolites, nitrite and nitrate, in Alzheimer's disease and vascular dementia of Binswanger type and multiple small infarct type, *J. Neural. Transm.*, 1998 **105**(10-12), 1283–1291 DOI 10.1007/s007020050131
- [16] Vandaveer IV, W.R.; Padas-Farmer, S.A.; Fischer, D.J.; Frankenfeld, C.N.; Lunte, S.M.: Recent developments in electrochemical detection for microchip capillary electrophoresis, *Electrophoresis*, 2004 **25**(21-22), 3528–3549 DOI 10.1002/elps.200406115
- [17] Mark, J.J.P., Scholz, R., Matysik, F.M.: Electrochemical methods in conjunction with capillary and microchip electrophoresis, *J. Chromatogr. A*, 2012 **1267**, 45–64 DOI 10.1016/j.chroma.2012.07.009
- [18] Kaniansky, D.; Masár, M.; Danková, M.; Bodor, R.; Rákociová, R.; Pilná, M.; Jöhnck, M.; Stanislawski, B.; Kajan, S.: Column switching in zone electrophoresis on a chip, *J. Chromatogr. A*, 2004 **1051**(1-2), 33–42 DOI 10.1016/j.chroma.2004.08.065
- [19] Kaniansky, D.; Masár, M.; Bodor, R.; Žúborová, M.; Ölvecká, E.; Jöhnck, M.; Stanislawski, B.: Electrophoretic separations on chips with hydrodynamically closed separation systems, *Electrophoresis*, 2003 **24**(1213), 2208–2227 DOI 10.1002/elps.200305474

# PHOSPHOGLYCOLIPID PROFILING OF BACTERIAL ENDOTOXINS

ANIKÓ KILÁR,<sup>1\*</sup> ÁGNES DÖRNYEI,<sup>2</sup> VIKTOR SÁNDOR,<sup>1</sup> FERENC KILÁR,<sup>1</sup> AND BÉLA KOCSIS<sup>3</sup>

1 Institute of Bioanalysis, University of Pécs, Szigeti út 12., Pécs, 7624, Hungary

2 Department of Analytical and Environmental Chemistry and Szentágotthai Research Center, University of Pécs, Ifjúság útja 6, Pécs, 7624, Hungary

3 Department of Medical Microbiology and Immunology, University of Pécs, Szigeti út 12., Pécs, 7624, Hungary

**Abstract** – Much interest is at present focused on bacterial endotoxins, also known as lipopolysaccharides (LPS), as they are responsible for the development of clinical symptoms of Gram-negative sepsis that is the leading cause of death in intensive care units. Endotoxicity is associated with the special phosphoglycolipid part of LPS, termed lipid A. Main challenges in the structure elucidation of lipid A arise from its amphiphilic character and inherent heterogeneity. We have developed a mass spectrometry based *de novo* method combined with reversed-phase liquid chromatography for the detailed structural characterization of complex lipid A mixtures (obtained by mild acid hydrolysis of LPS) from different bacterial sources. Tandem mass spectrometric measurements were performed with an electrospray quadrupole time-of-flight (ESI-Q-TOF) mass spectrometer in both negative- and positive-ionization modes in order to explore fragmentation pathways. We found that characteristic product ions in the positive-ion mode could be used for the unambiguous assignment of the phosphorylation site, whereas the use of both ionization modes provided consistent and/or complementary information about the fatty acyl distribution between the two glucosamine moieties of lipid A. Since the immunostimulatory (advantageous) vs. proinflammatory (endotoxic) effect of the lipid A is in close relation with the fine chemical structure, our relatively simple structure elucidation strategy could offer great potential in the bioanalysis of native lipid A samples and lipid A-based vaccines.

**Keywords:** lipid A, HPLC, tandem mass spectrometry, positive-ion mode, negative-ion mode

## 1. Introduction

Bacterial endotoxins are important initiators of clinical symptoms of Gram-negative sepsis, a serious medical condition with high mortality rate among patients in intensive care units worldwide. Endotoxins, chemically lipopolysaccharides (LPS) or lipooligosaccharides (LOS) are found on the surface of Gram-negative bacteria. They are essentially composed of two regions: a heteropolysaccharide chain (divided into the oligosaccharide core and the O-side chain – this component is missing from LOS) that extends outwards from the bacterial cell surface, and a phosphoglycolipid, termed lipid A, which is anchored in the outer membrane (*Fig.1*). Specifically, the endotoxic activity is related only to the lipidic domain of endotoxins.

In respect of toxicity of lipid A the crucial factors are the acylation pattern and phosphorylation status. The most biologically potent molecule, capable of inducing septic shock, consists of an assymmetrically hexa-acylated, bisphosphorylated glucosamine disaccharide [1]. Other lipid A species with deviations from this structure have lower endotoxicity or may even

have beneficial effects. Consequently, the structure analysis of the lipid A moiety is of fundamental interest in clinical aspects and vaccine development.

The structural characterization of lipid A poses a unique analytical challenge, as lipid A isolated from a single bacterial strain generally consists of a mixture of differently acylated and phosphorylated species. Furthermore, the relative abundances of the different structures can vary with growth conditions (*e.g.*, temperature, culture media, pH). Within the field, tandem mass spectrometry (MS/MS or MS<sup>n</sup>) has emerged as a core technology for the in-depth structural elucidation of lipid A species [2].

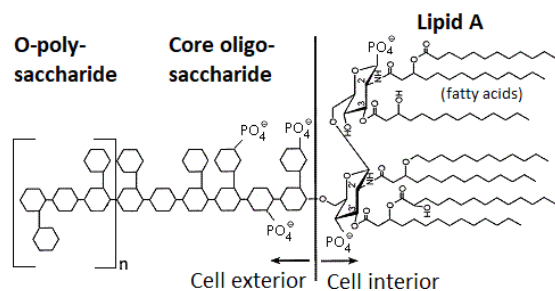


Figure 1. Schematic representation of the chemical structure of an enterobacterial outer membrane lipopolysaccharide (endotoxin).

However, separation and simultaneous quantification of singular components by powerful separation methods prior to MS would be highly desirable to adequately characterize the heterogenic structures.

Here, we give a brief presentation of an alternative method to multiple-stage MS for the differentiation of various lipid A species – including isobars – within a bacterial sample. Our method is based on a reversed-phase HPLC separation and online ESI-Q-TOF mass spectrometric detection of molecular species in native lipid A isolates. The exact structures of the lipid A molecules in the complex mixtures were deduced from specific fragmentation patterns obtained in both, the negative- [3,4] and the positive-ion modes [5].

## 2. Experimental

### 2.1. Chemicals and Reagents

Methanol, isopropyl alcohol, water (LC-MS grade), dichloromethane (HPLC,  $\geq 99.9\%$ ), triethylamine ( $\text{Et}_3\text{N}$ ), acetic acid (AcOH) were purchased from Sigma-Aldrich (Steinheim, Germany).

### 2.2. Bacterial strains and lipid A isolation

*Escherichia coli* O111 and *Proteus morganii* O34 strains were cultured in a laboratory fermentor, and then collected by centrifugation. The bacterial LPS was extracted from acetone-dried organisms by the traditional hot phenol/water procedure and lyophilized.

Lipid A was released from LPS by mild-acid hydrolysis with 1% (v/v) AcOH at 100°C for 1 h, then the solution was centrifuged (8000 $\times$ g, 4°C, 20 min). The sediment (containing lipid A) was washed four times with distilled water and lyophilized. Lipid A samples were dissolved in methanol/dichloromethane (95:5, v/v) at a final concentration of 0.5 mg/ml.

### 2.3. Liquid Chromatography – Mass Spectrometry Measurements

Separation and mass spectrometric detection of lipid A samples were performed with an Infinity 1290 UHPLC system (Agilent Technologies, Waldbronn, Germany) coupled to an 6530 Accurate Mass Q-TOF LC/MS system (Agilent Technologies, Singapore), using a core-shell Kinetex™ C18 column (100 mm $\times$ 4.6 mm, 2.6  $\mu\text{m}$  particle size, Phenomenex). Mobile phase A was methanol/water (95:5, v/v) and mobile phase B was isopropyl alcohol, both containing 0.1%  $\text{Et}_3\text{N}$  and 0.1% AcOH (v/v). Injection volume was 3  $\mu\text{l}$ . Separation was performed at a constant flow rate of 0.5 ml/min at 50°C. A linear gradient of 50 min started at 0% mobile phase B and ramped to 50% mobile phase B, and then hold at 50% mobile phase B for 2 min.



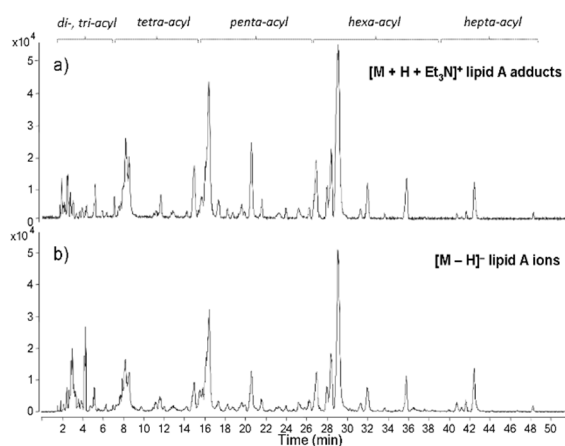


Figure 2. HPLC–MS base peak chromatograms of the native, heterogeneous *E. coli* O111 lipid A preparation acquired in the a) positive- and b) negative-ion modes.

Negative- and positive-ion mass spectra of the column eluate were recorded between  $m/z$  100–3200. The ESI ion source was operated using the following conditions: pressure of nebulizing gas ( $N_2$ ), 30 psi; temperature and flow rate of drying gas ( $N_2$ ), 325°C and 8 l/min, respectively; temperature and flow rate of sheath gas, 300°C and 11 l/min, respectively; capillary voltage, 4 kV; nozzle voltage, 2 kV; fragmentor potential, 100 V; skimmer potential, 70 V. For the targeted MS/MS analysis, the following parameters were used: mass range,  $m/z$  70–2000; acquisition rate, 333.3 ms/scan; quadrupole isolation width, 1.3  $m/z$ ; collision energy, 70–100 V; maximum precursor ions/cycle, 4; precursor ion active charge state, 1.

### 3. Results and Analysis

#### 3.1. HPLC-MS of bacterial lipid A

Crude lipid A preparations from *E. coli* O111 and *P. morgani* O34 bacteria were used as training sets for the method development. The HPLC separation conditions were optimized in order to find a balance between solubility, adequate separation and ESI ionization of the intact lipid A's. Fig.2 shows the HPLC–MS analysis in the positive- and negative-ion modes for the *E. coli* O111 lipid A isolate, only. We found that the lipid A samples expressed extreme structural heterogeneity, showing the presence of numerous bis-, mono-, and non-phosphorylated structures

and diverse acylation states ranging from di- to hepta-acylation. The most abundant chromatographic peak from both bacteria was a hexa-acylated, monophosphorylated species. The elution order of the separated compounds was consistent with their relative hydrophobicity, defined mainly by the total number and length of hydrophobic acyl chains, followed by the number of polar phosphate groups. Several isomers with the same masses could be identified, in which the length and/or position of the acyl chains varied.

Since the phosphoglycolipids have acidic character, they could readily be ionized as deprotonated ions, *i.e.*,  $[M - H]^-$  in negative-ion mode, whereas their ionization in the positive-ion mode was enhanced by adduct formation with triethylamine used as an eluent additive, *i.e.*,  $[M + H + Et_3N]^+$  adducts were formed.

#### 3.2. Tandem mass spectrometric analysis of lipid A species

In order to investigate the fragmentation behavior under low-energy collision-induced dissociation (CID) conditions of each separated lipid A precursor ion, ESI-MS/MS experiments were performed in both ionization modes. Based on previously published data on the acyl distribution of the C4'-monophosphorylated, hexa-acylated lipid A molecule from *E. coli* O111 [6], ions observed in the tandem mass spectra (not shown) of this lipid A species (appearing at  $t_R = 29.2$  min in Fig.2) could be structurally assigned in both ionization modes [3,5]. Using this as a standard, we have compared the CID mass spectral profiles of other mono-phosphorylated lipid A species separated by the chromatography and explored the fragmentation preferences for each of them, in both ionization modes. Fig.3 shows an example for the correlation between the phosphoglycolipid structure and the fragmentation profiles in the case of the main hepta-acylated lipid A compound ( $t_R = 42.5$  min in Fig.2) from *E. coli* O111. The site specific cleavages are assigned with a number denoting the carbon of the glucosamine to which the substitution is attached, and a Greek letter designating the position of the bond which was cleaved.

In the tandem mass spectrum of the  $[M + H + Et_3N]^+$  triethylammonium adduct of this lipid A

species (Fig.3a), there were only a few ions resulting from the neutral loss of acyl chains, however, in that of the deprotonated form (Fig.3b), the abundances of product ions in the higher  $m/z$  region straightforwardly indicated the consecutive dissociation order of the ester-linked fatty acids. Thus their position could be assigned. Each tandem mass spectrum also showed diagnostic intra-ring (A-type ions in the negative-ion mode) or inter-ring (B- and Y-type ions in the positive-ion mode) cleavage products, which confirmed the substitution position of the fatty acids on the two glucosamine residues.

Fig.4 shows the tandem mass spectra of the C1 phosphorylation isomer of this hepta-acylated lipid A. The location of the single phosphoryl group (i.e., at C1 or C4') could be directly assessed from the positive-ion mode tandem mass spectra by calculating the mass difference between the  $m/z$  of the precursor ion and that of the product ion resulting from the cleavage of the 1 $\alpha$  bond (compare Figs.3a and 4a). Namely, the mass difference of 199 u corresponded to the combined loss of triethylamine and an orthophosphoric acid molecule, indicating that a phosphoric acid had esterified the C1 hydroxyl group of the reducing end. On the other hand, a mass difference of 119 u resulted from the combined loss of triethylamine and a water molecule, indicating the presence of a free hydroxyl group at C1 (consequently, the phosphoryl group must be at C4').

The change in the phosphorylation site also resulted in a distinctive tandem mass spectrum in the negative-ion mode (Fig.4b). Main differences were the complete -

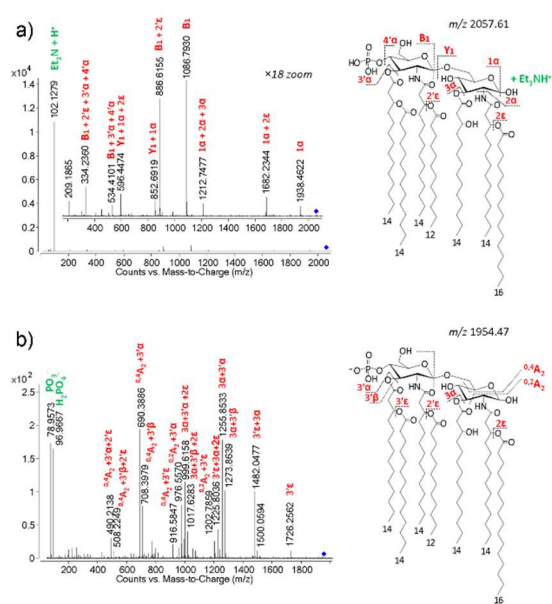


Figure 3. Tandem mass spectra in a) positive- and b) negative-ion modes and depiction of the identified structure of the C4'-monophosphorylated hepta-acylated lipid A from *E. coli* O111 bacterium. Site-specific cleavages are indicated next to the ion signals.

lack of A-type cross-ring fragments, and instead the appearance of Z-type inter-ring cleavage products. Furthermore, the intensity of the product ions resulting from the neutral loss of acyl chains were about the same magnitude, indicating a strong competition between the fatty acyl eliminations during the CID process, instead of their stepwise dissociation (compare with Fig.3b).

The fragmentation behavior of non- and bis-phosphorylated lipid A species under both, negative and positive CID conditions were investigated, as well. For these, a different sequencing of the acyl-chain cleavages could be observed in the negative-ion mode [3,4], whereas they showed similar fragmentation pathways to that of the monophosphorylated ones in the positive-ion mode [5]. Besides the A-type ions (nonreducing end), complementary X-type fragment ions (reducing end) appeared in the negative-ion mode tandem mass spectra of the nonphosphorylated lipid A's [4].

## 4. Discussion

The online HPLC–MS/MS methodology enabled the simultaneous structural characterization of both, phosphorylated and nonphosphorylated lipid A variants, within a single run. The main advantage of using the collision cell of a Q-TOF mass spectrometer was that several generations of precursor/product ions – that would otherwise be generated only at higher MS stages with ion trap experiments – were observed at the same time in the tandem mass spectra of the separated lipid A species. This allowed us to set new fragmentation rules for the differently phosphorylated and acylated lipid A compounds by a simple MS/MS experiment [3-5]. Specifically, the acylation profile of the non-, C4'-mono, and bisphosphorylated lipid A could be inferred partly from the positive- and fully from the negative mode MS/MS analysis [3-5], whereas the complementary use of both ionization modes [3,5] was needed for the full structural characterization of C1-monophosphorylated lipid A.

As an example, the complete structure elucidation strategy by MS/MS of a hepta-acylated C1-monophosphorylated lipid A is the following. First, the observation of the 1 $\alpha$  cleavage product in the positive mode is essential to clarify the site of the phosphorylation (*vide supra*). Next, the linkages at the 2' $\epsilon$  (only from the B-type ion) and 2 $\epsilon$  positions can be revealed from the positive mode analysis. By knowing the fragmentation preference of the secondary ester bonds (which is 2' $\epsilon$ >3' $\epsilon$ >2 $\epsilon$  [5]), substitutions at these sites can be deconvoluted from the negative mode analysis by observing the 2' $\epsilon$ , 3' $\epsilon$ , 2 $\epsilon$ , and 3 $\alpha$  cleavage products in the upper  $m/z$  region and those resulting from the 3' $\alpha$ , 2' $\epsilon$ 3' $\epsilon$ , 2' $\epsilon$ 2 $\epsilon$ , 2' $\epsilon$ 3 $\alpha$ , and 3' $\epsilon$ 3 $\alpha$  cleavages, displayed in the middle  $m/z$  region. Here, the dephosphorylated Z-type ion in the lower  $m/z$  region indirectly specifies the linkage at the 2 $\alpha$  position. Finally, the remaining 2' $\alpha$  substitution can be deduced from the intact B-type ion (positive-ion mode).

## 5. Conclusion

An efficient HPLC separation before the MS detection of natural lipid A mixtures has proven

particularly useful to reveal the diversity and the relative amount of the various lipid A structures present in bacteria. Such investigations could help to explore the relative contributions of different lipid A structures to the overall activation of the innate immune system. Moreover, knowledge of the chemical and biological aspects of lipid A heterogeneity is crucial in order to design antimicrobial drugs which overcome the evasion strategy of the pathogen. Our technique could be well suited for phosphoglycolipid profiling from different bacterial strains or vaccine preparations.

## Acknowledgement

The research was supported by the grants OTKA K-125275 and ÚNKP-17-4-III New National Excellence Program of the Ministry of Human Capacities.

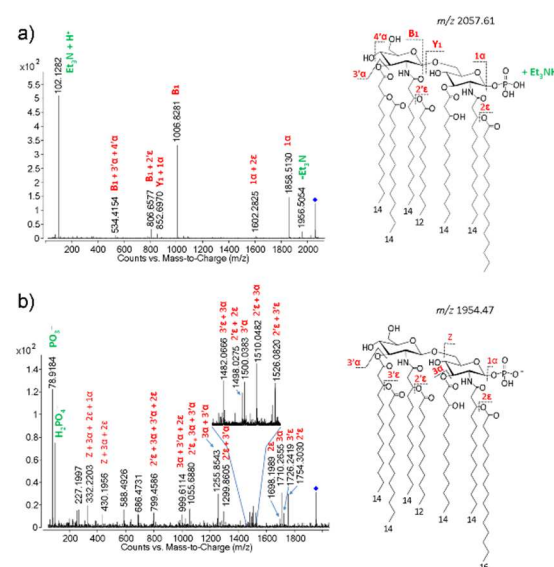


Figure 4. Tandem mass spectra in the a) positive- and b) negative-ion modes and depiction of the identified structure of the C1-monophosphorylated hepta-acylated lipid A from *E. coli* O111.

## REFERENCES

- [1] Munford, R.S.: Sensing gram-negative bacterial lipopolysaccharides: a human disease determinant? *Infect. Immun.* 2008, **76**(2), 454–465. DOI: 10.1128/IAI.00939-07
- [2] Kilár, A.; Dörnyei, Á.; Kocsis, B.: Structural characterization of bacterial lipopolysaccharides

- with mass spectrometry and on- and off-line separation techniques. *Mass Spectrom. Rev.* 2013, **32**(2), 90–117. DOI: 10.1002/mas.21352
- [3] Sándor, V.; Dörnyei, Á.; Makszin, L.; Kilar, F.; Péterfi, Z.; Kocsis, B.; Kilar, A.: Characterization of complex, heterogeneous lipid A samples using HPLC-MS/MS technique I. Overall analysis with respect to acylation, phosphorylation and isobaric distribution. *J. Mass Spectrom.* 2016 **51**(11), 1043–1063. DOI: 10.1002/jms.3839.
- [4] Sándor, V.; Kilar, A.; Kilar, F.; Kocsis, B.; Dörnyei, Á.: Characterization of complex, heterogeneous lipid A using HPLC-MS/MS technique II. Structural elucidation of non-phosphorylated lipid A by negative-ion mode tandem mass spectrometry. *J. Mass Spectrom.* 2016, **51**(8), 615–628. DOI: 10.1002/jms.3786
- [5] Sándor, V.; Kilar, A.; Kilar, F.; Kocsis, B.; Dörnyei, Á.: Characterization of complex, heterogeneous lipid A samples using HPLC-MS/MS technique III. Positive-ion mode tandem mass spectrometry to reveal phosphorylation and acylation patterns of lipid A. *J. Mass Spectrom.* 2017 – submitted for publication
- [6] Madalinski, G.; Fournier, F.; Wind, F.L.; Afonso, C.; Tabet, J.C.: Gram-negative bacterial lipid A analysis by negative electrospray ion trap mass spectrometry: stepwise dissociations of deprotonated species under low energy CID conditions. *Int. J. Mass Spectrom.* 2006, **249–250**, 77–92. DOI: doi:10.1016/j.ijms.2005.12.049

# MINIATURIZED DEVICE FOR SINGLE-CELL ANALYSIS OF CASPASE-3 AND 7 ACTIVATION USING BIOLUMINESCENCE CHEMISTRY

VOJTĚCH LEDVINA<sup>1,2</sup> AND KAREL KLEPÁRNÍK<sup>1</sup>

1 Department of Bioanalytical Instrumentation, Institute of Analytical Chemistry of the Czech Academy of Sciences, v. v. i., Veveří 967/97, Brno, 602 00, Czech Republic

2 Faculty of Science, Masaryk University, Kamenice 753/5, Brno, 625 00, Czech Republic

**Abstract** – Caspases are proteases that play key role in the process of apoptosis, the programmed cell death. Among them, caspase-3 and -7 are main executioner caspases that cleave many vital proteins during apoptosis and after their widespread activation, the process cannot be reversed. To analyze caspase-3/7 activation within single cells, a miniaturized device for parallel analysis of eight samples was developed. The assay is based on the modified luciferin-firefly luciferase bioluminescence (BL) system. Individual suspended cells were collected and transferred into detection microvials using a micromanipulator. The bioluminescence was detected using a photon counting head with cooled photocathode. The LOD suitable for detection of active caspase-3/7 in both individual apoptotic and non-apoptotic cells was reached.

**Keywords:** bioluminescence, single-cell analysis

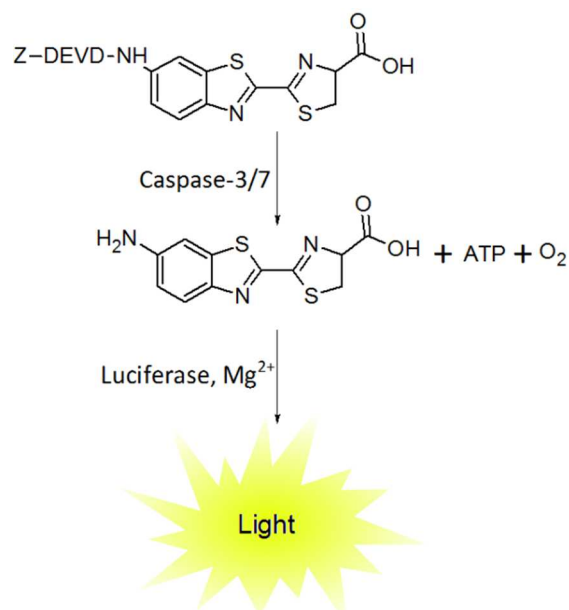
## 1. Introduction

Caspases are key proteolytic enzymes in the process of apoptosis – the programmed cell death. The executioner caspases are the main proteolytic machinery that ensures an organized and clean deconstruction of unwanted cells without damaging surrounding cells and eliciting inflammatory response. Among them, caspase-3 and -7 are the most effective and their role in apoptosis makes them one of many potential targets in cancer treatment. Besides their apoptotic functions, new information is being revealed about their role in nonapoptotic events as well. Their activity has been detected for example during skeletal muscle differentiation, osteogenesis or brain development [1].

To study the caspase-3/7 content at the single-cell level, sensitive and specific method of detection is a necessary prerequisite for successful analysis. In our study, we have utilized a commercially available bioluminescence (BL) assay CaspaseGlo® 3/7 (Promega) which is based on the modified aminoluciferin-firefly luciferase system [2]. The aminoluciferin is conjugated with Asp-Glu-Val-Asp (DEVD) peptide specifically cleaved by caspase-3/7 which prevents it from being oxidized by the luciferase. In the presence of caspase, the peptide group is cleaved off, the aminoluciferin is oxidized and a steady flow of photons is generated as shown in Fig. 1. The enzymatic nature of the BL reaction provides excellent sensitivity thanks to the

fact that single caspase molecule can cleave multiple aminoluciferin molecules and the background signal is very low due to the absence of excitation light and interfering autofluorescence.

In our study, a miniaturized device for parallel BL detection of active caspase-3/7 content in single apoptotic and non-apoptotic cells was developed.



*Fig. 1* Schematic depiction of the BL reaction for determination of caspase-3/7. Aminoluciferin can be oxidized by luciferase only after the caspase-3/7 specific peptide is cleaved off.

The device is compatible with analyses of suspended cells selected and transferred by a micromanipulator mounted on a microscope. Our objective was to reach LOD low enough to detect active caspase-3/7 in single non-apoptotic cells in

\*\*Correspondence: klep@iach.cz

order to study caspase role during cell differentiation. To accomplish this, a sensitive assembly of photomultiplier tube (PMT) with a cooled photocathode working in the photon counting mode was implemented.

## 2. Experimental

### 2.1. Cell culture and preparation

Micromass cultures isolated from mouse forelimbs at embryonic day 12 were selected as a model. Cells were cultured in a medium consisting of DMEM and DME F12 media supplemented with 10% fetal bovine serum, 0.5mM glutamine, 1mM  $\beta$ -glycerolphosphate, 0.25mM ascorbic acid and 25 U/mL of penicillin and 25  $\mu$ g/mL streptomycin. Two experimental groups were

prepared: a control group cultivated in normal culture medium (non-apoptotic cells) and an experimental group treated with 5  $\mu$ M camptothecin for 8 hours (apoptotic cells). Before analysis, cells were trypsinized using 350  $\mu$ L of 0.25% trypsin at 37 °C for 5 minutes and diluted in the growth medium.

### 2.2. Cell collection and measurements

All measurements were done in 5  $\mu$ L microvials that were made of glass capillaries with 1.3/1.9 mm i.d./o.d. by fusing at one end and cutting at the other. Each microvial was filled with 4  $\mu$ L of the CaspaseGlo® 3/7 reagent and placed into a plastic microvial holder. Cells were collected from the trypsinized cell suspension using the ICSI micromanipulator for *in vitro* fertilization (Eppendorf) mounted on an inverted microscope Olympus IX71 and equipped with a holding pipette with 20  $\mu$ m inlet diameter. One to six cells were collected from a cell suspension at a time and transferred into individual detection microvials (Fig. 2).

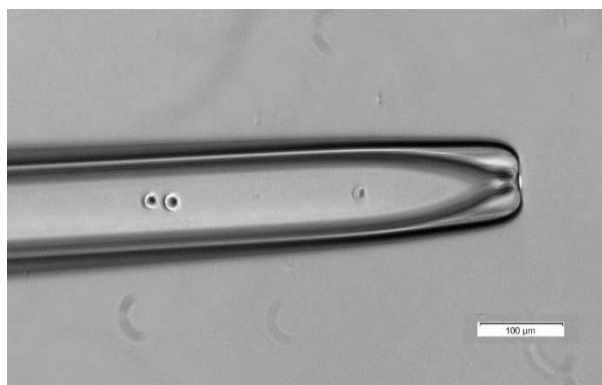


Fig. 2 Holding capillary with three murine cells inside. The scale bar is 100  $\mu$ m.

After filling, all eight microvials were placed into a stainless steel revolving carousel vial holder and the whole assembly was mounted in front of the PMT detection window (Fig. 3). The carousel has a set of grooves, each for an individual microvial, with polished inner surface that reflects scattered photons to the PMT detection window. The BL emission leaving individual vials was detected consecutively and repeatedly in 5 min intervals for a period of about 80 minutes until the steady state was reached in all vials.

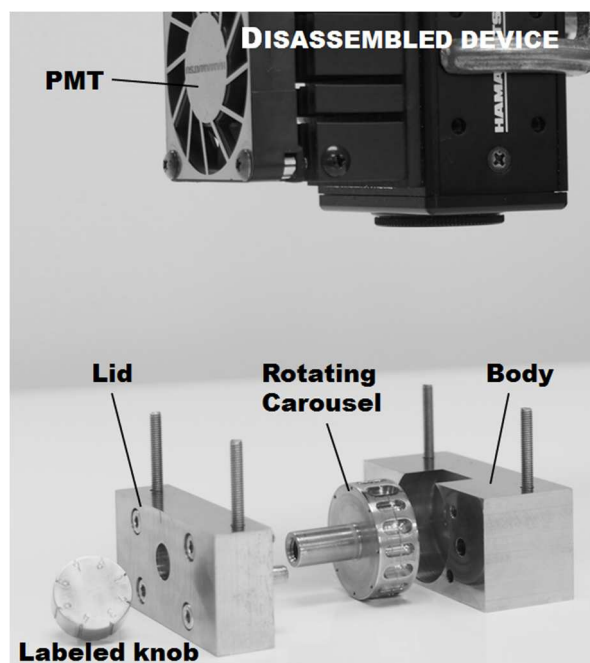


Fig. 3 Disassembled detection device consists of a stainless steel carousel with grooves with polished inner surface that reflect maximum of the emitted photons towards the photomultiplier photocathode. Two sets of grooves are present – 8 positions for 5  $\mu$ L microvials and 8 positions for larger 10  $\mu$ L vials. When the device is assembled, only one microvial is facing the PMT window at a time.

The detection assembly consisted of a photon counting head H7421-40 series with a heat sink and fan A7423, counting unit C8855 01 and power supply unit with temperature control M 9011 (all Hamamatsu Photonics, Japan). The photon counting head is furnished with a PMT having a GaAsP/GaAs photocathode and a thermoelectric cooler. The cooler maintains PMT at a constant temperature of 0 °C and reduces thermal noise generated by the photocathode, thus increasing S/N ratio.

### 3. Results and discussion

From a biological point of view, it is important to study the role of caspases at a single-cell level because bulk measurements from a large sets of cells obscure the cell-to-cell variations and the results are averaged throughout the whole population. Therefore, sensitive methods are necessary. One of the prerequisites of high sensitivity analyses of ultra-low volume samples is miniaturization resulting in reduction of background signal and chemicals consumption. In the case of CaspaseGlo® 3/7 reagent, the main contribution to the background signal is the accidentally released aminoluciferin that is accessible for luciferase oxidation. Its total amount linearly increases with the volume of the reagent. On the other hand if the volume of the reagent is too low, modified luciferin can be depleted before reaching the BL steady state, thus decreasing the actual signal. To reach the signal maximum, we used detection microvials of 1.3 mm i.d. filled with 4 µl of the reagent. This was enough to provide sufficient substrate delivery to the

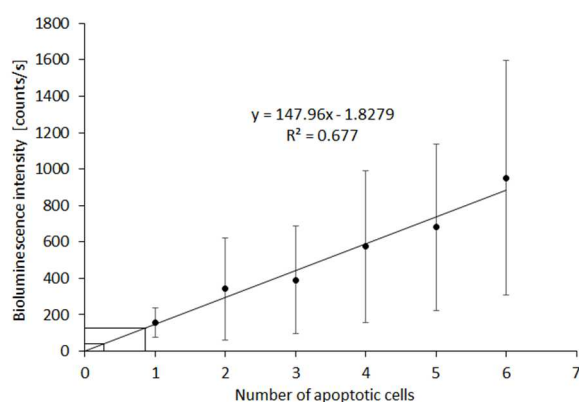


Fig. 4 Dependence of BL signal on the number of collected apoptotic cells. The LOD and LOQ are below the average content of caspase in a single apoptotic cell.

active caspase-3/7 molecules while maintaining relatively low background signal. Further decrease of the background was achieved by using a PMT with cooled photocathode. The photocathode yields a dark current of only about 3-6 photon counts/s which is very low compared to the blank signal of the reagent (about 62 counts per second).

The device was tested with apoptotic and non-apoptotic cells. No cross-talk was observed even when neighbouring microvials had a big differences in the bioluminescence intensity. In both cases, the caspase-3/7 content linearly increased with the number of cells. The average signal intensity for apoptotic cells ranged from 157-952. The average signal intensity for apoptotic cells ranged from 157-952 counts/s and was approximately three times higher than for the non-apoptotic cells where the signal ranged from 56-241 counts/s. High standard deviations were apparent particularly in the case of apoptotic cells. The deviations can be caused by cell heterogeneity, by the aspects of dynamics of camptothecin induced caspase activation in individual cells and the effect of the cell cycle phase of individual cells. It is also possible that camptothecin may not diffuse properly to the lower layers of the cells in Petri dishes that adhere directly to the surface during cultivation since cells in micromass cultures do not grow in a single layer. Some (very low) activation of caspase-3/7 in non-apoptotic cells can be due to the *ex vivo* approach and micromass treatment. It may also suggest some physiological basal level of activation or can even point to possible non-apoptotic functions of these caspases.

The LOD and LOQ were calculated as three and ten times the standard deviation of a blank, respectively. For apoptotic cells the LOD/LOQ were evaluated to be 0.27/0.86 of a caspase-3/7 content in an average apoptotic cell and for the non-apoptotic cells 0.46/2.92 of a caspase-3/7 content in an average non-apoptotic cell. The device is therefore suitable for both detection and quantification of active caspase-3/7 in individual apoptotic cells. In the case of non-apoptotic cells, caspase-3/7 can be detected but not reliably quantified.

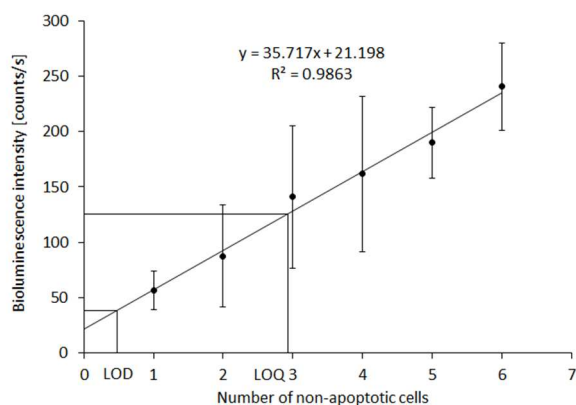


Fig. 5 Dependence of BL signal on the number of collected nonapoptotic cells. The LOD value is below the average content in a single non-apoptotic cell while the LOQ is higher and needs at least 3 cells for reliable quantification.

The device was also tested with laser capture microdissection where an area of cells of interest within a tissue can be specifically dissected by laser beam and catapulted into the vials containing the reagent. When compared with a tissue area that is known to not contain active caspases, one can relatively compare activity between the sample and control and perform localized studies of the enzyme activation during e.g. tooth or bone development.

#### 4. Conclusion

A miniaturized device for sensitive detection of caspase-3 and 7 was developed with the LOD below the average content of active caspase-3/7 in both single apoptotic and non-apoptotic cells. The LOQ is low enough to allow caspase-3/7 quantification in individual apoptotic cells that generally contain higher amount of active proteases. In the case of normal non-apoptotic cells, at least three cells have to be gathered into the microvial for reliable quantification. The weakest point of the device at the moment is the limited throughput because only eight samples can be analyzed in parallel. The throughput could be potentially improved by using a microplate from a black plastic that would eliminate cross-talk between individual wells combined with a fast and effective system for cell collection and transfer. This would allow acquisition of larger and more statistically significant datasets.

#### Acknowledgement

The research was supported by Grant Agency of the Czech Republic, project No. 17-01995s and by institutional support RVO 68081715 of the Institute of Analytical Chemistry, Czech Academy of Sciences, v.v.i.

#### REFERENCES

- [1] Shalini S., Dorstyn L., Dawar S., Kumar S.: Old, new and emerging functions of caspases. *Cell Death Differ*, 2015 **22(4)**, 526-539.
- [2] O'Brien M.A., Daily W.J., Hesselberth P.E., Moravec R.A., Scurria M.A., Klaubert D.H., Bulleit R.F., Wood K.V.: Homogeneous, bioluminescent protease assays: Caspase-3 as a model. *J Biomol Screen*, 2005 **10(2)**, 137-148.



# DEVELOPING OF A HIGH-RESOLUTION SEPARATION GEL FOR PROTEIN ANALYSIS BY CGE-MS

BRIGITTA MÉSZÁROS<sup>1,2</sup>, SZIGETI MÁRTON<sup>1,2</sup>, GUTTMAN ANDRÁS<sup>1,2</sup>

1 Horváth Csaba Memorial Institute of Bioanalytical Research, University of Debrecen

2 MTA-PE Translational Glycomics Research Group, Research Institute for Biomolecular and Chemical Engineering, University of Pannonia, Veszprem, Hungary

## Abstract

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has been the method of choice for size-based separations of proteins for over decades and plays a well-established role in protein separations and analyses in most of the biological research laboratories. However, in recent years SDS-capillary gel electrophoresis (SDS-CGE) also gained significant importance in bioanalytics, especially in the biopharmaceutical industry. SDS-CGE can be considered as the automated version of SDS-PAGE, having numerous advantages such as on-column detection, automated operation, high resolving power, and capability of accurate protein quantification as well as molecular mass determination. In the biopharmaceutical industry SDS-CGE has been recognized as an important tool in process development and quality control of proteins and glycoproteins.

SDS-CGE hyphenation with mass spectrometry (MS) analysis offers additional selectivity provided by the MS spectrum. However, SDS-CGE coupling with MS is very challenging since the separation gel and the surfactant (SDS) can contaminate the MS and may compromise its detection system. Our goal was to develop an MS friendly separation buffer and gel system for protein analysis. Major aspects, which have been taken into consideration during the development process were 1) separation gel components have to be compatible with mass spectrometer and 2) avoid non MS compatible compounds getting into the MS. The determination of an optimal protein separation gel-buffer composition was the major focus of my work with special emphasis on MS compatibility.

## Acknowledgment

The authors gratefully acknowledge the support of the Momentum (Lendulet) grant # 97101 of the Hungarian Academy of Sciences (MTA-PE Translational Glycomics) and the National Research, Development and Innovation Office (NKFIH) (K 116263) grants of the Hungarian Government. This work was also supported by the BIONANO\_GINOP-2.3.2-15-2016-00017 project.

# CHARACTERIZATION OF A MICROFLUIDIC ENZYME REACTOR FOR THE RAPID PROTEOLYSIS OF HUMAN TEAR SAMPLES

CYNTHIA NAGY, ADAM KECSKEMETI, ATTILA GASPAR

Department of Inorganic and Analytical Chemistry, University of Debrecen, H-4032 Debrecen, Egyetem tér 1, Hungary

## Abstract

In our research we created a microfluidic chip capable of rapid and efficient proteolysis of tear samples [1]. The chip-based digestion was compared to the standard, in-solution digestion to investigate the suitability of the newly developed microchip for clinical samples. The in-solution digestion procedure includes the addition of trypsin to the sample in a low concentration to limit undesired autolysis. However, this results in long incubation times, which hinders the fast, high-throughput analysis of our peptide mixture. The problem of the time-consuming in-solution digestion was solved with the use of microfluidic chips. The microchips were fabricated by means of soft photolithography [2] from poly(dimethylsiloxane) (PDMS), which enables the strong adsorption of proteins on its surface. Utilizing the porous feature of PDMS, trypsin was immobilized on the channel walls of the microchip through spontaneous adsorption. The surface-bound trypsin was applied in a high concentration, thus digestion time was greatly reduced with the use of microchips (50 s) compared to those typical of in-solution digestions (16 h). Owing to the design of the chip, high throughput sample processing was made possible, up to 8 digestions could be carried out simultaneously. The digested samples were analyzed using capillary zone electrophoresis (CZE). The obtained results clearly indicate the applicability of the designed microchip for reliable tear-sample proteolysis. In addition, further attempts to optimize protein digestion by increasing the surface to volume ratio in the microchips are underway, which would make the process not only faster, but also more effective.

**Keywords:** proteomics, peptide mapping, capillary zone electrophoresis, microfluidics, immobilization

## References:

- [1] A. Kecskemeti, C. Nagy, E. Csoz, G. Kallo, A. Gaspar, The application of a microfluidic reactor including spontaneously adsorbed trypsin for rapid protein digestion of human tear samples. *Proteomics-Clin. Appl.* DOI 10.1002/prca.201700055
- [2] Whitesides, G.M., Ostuni, E., Takayama, S., Jiang, X., Ingber, D.E., Soft lithography in biology and biochemistry. *Annu. Rev. Biomed. Eng.* 2001, 3, 335–373.

Correspondence: gaspar@science.unideb.hu

Below is the article on which my planned presentation is based:

<http://onlinelibrary.wiley.com/doi/10.1002/prca.201700055/full>

# COMPARISON OF FOUR METHODS USED FOR PLASMA PROTEIN-DRUG INTERACTION STUDIES

HANA NEVÍDALOVÁ\*, LENKA MICHALCOVÁ, ZDENĚK GLATZ

Department of Biochemistry, Masaryk University, Kamenice/5, Brno, 625 00, Czech Republic

Abstract – Interaction of drugs with plasma proteins influence drug liberation, adsorption, disposition, metabolism, elimination and toxicological properties. Thus insight into plasma protein-drug binding is essential for predicting clinical dosages and assessment of safety boundary of drugs. In this study binding parameters of human serum albumin-diclofenac and human serum albumin-lidocaine pairs were determined by four methods – capillary electrophoresis-frontal analysis, isothermal titration calorimetry, circular dichroism and equilibrium dialysis. All these approaches were compared in detail and their benefits and drawbacks were demonstrated.

**Keywords:** Capillary electrophoresis-frontal analysis, isothermal titration calorimetry, circular dichroism, equilibrium dialysis

## 1. Introduction

Plasma protein-drug binding influence pharmacodynamic and pharmacokinetic properties of the drugs. It is widely known that plasma proteins transport bound drugs in the blood but simultaneously these interactions thus limit drug concentration available to provide pharmacological effect as well as drug disposition and efficacy [1]. For this reasons the study of these interactions is an essential part in development of an new drugs. Requirement to increase laboratory efficiency leads to development of an automated high-throughput assays [2].

Human serum albumin (HSA) and  $\alpha$ 1-acid glycoprotein are the most important proteins with respect to drug binding. HSA carries mostly acidic drugs,  $\alpha$ 1-acid glycoprotein is the carrier for basic and neutral drugs [3]. In this consequence the HSA was chosen as model protein in this study. HSA is synthesized in the liver by hepatocytes and further modified in Golgi apparatus. It is the most abundant protein in blood plasma with concentration 35-45 g/L, its half-life is 19 days, molecular mass 66 500 Da and isoelectric point 4.8. HSA consists of a single polypeptide chain of 585 amino acids and its secondary structure is predominantly composed of an  $\alpha$ -helix. This protein can be divided into three homologous helical domains and each of these domains contains two subdomains A and B [4].

In this study four methods capillary electrophoresis-frontal analysis (CE-FA), isothermal titration calorimetry (ITC), circular dichroism (CD) and equilibrium dialysis (ED) used for measuring the binding parameters of the model systems HSA-

diclofenac and HSA-lidocaine were complexly compared. Used drugs were chosen based on their different binding strength and physico-chemical properties.

All measurements were performed with the same buffer (ionic strength, pH), protein concentration and temperature.

## 2. Experimental

### 2.1. Samples and Measurements

Boric acid, diclofenac (DCF), HSA, lidocaine (LIDO), NaOH and sodium tetraborate decahydrate were obtained from Sigma Aldrich (Steinheim, Germany), HCl was obtained from Fluka (Buchs, Switzerland). All chemicals used were of analytical grade purity or the best purity available.

The borate buffer used in all experiments was prepared by mixing 200 mM boric acid and 150 mM sodium tetraborate, the pH of the final solution was 8.5. The pH values were measured with an Orion Research EA940 pH meter (Orion Research Waltham, MA, USA). The HSA and drug samples were prepared by dissolution in this buffer. All solutions were prepared using water from a Millipore Direct Q 5 UV system (Merck, Milford, MA, USA).

#### 2.1.1. CE-FA measurements

A new capillary was conditioned by rinsing with 1 M NaOH for 20 min, then with deionised water for 10 min and finally with the BGE for 20 min. At the beginning of each day, the capillary was

\*Correspondence: h.nevidalova@seznam.cz

conditioned by rinsing with 1 M HCl for 10 min, deionized water for 5 min, 1 M NaOH for 10 min, deionized water for 5 min and the BGE for 20 min. Before each analysis the capillary was flushed for 1 min with 1 M HCl, for 1 min with deionized water, for 2.5 min with 1 M NaOH, for 1 min with deionized water and for 2 min with BGE. All rinsing steps were done with a pressure of 950 mbar (95 kPa).

The bare fused silica capillary 75  $\mu\text{m}$  I.D. (58.5/50 cm  $L_{\text{tot}}/L_{\text{eff}}$ ) was thermostated at 25  $^{\circ}\text{C}$ , samples were injected at 35 mbar (3.5 kPa) for 40 s and an operating voltage of 14 kV was applied in positive polarity mode. Detection wavelengths were set to 276 nm for DCF and 214 nm for LIDO, respectively. Standards of drug samples (20 - 800  $\mu\text{M}$ ) were used to prepare the calibration curve and to validate the CE-FA method. For the binding experiments, a series of samples containing a constant concentration of HSA (75  $\mu\text{M}$ ) and varying concentrations of drugs (50 - 800  $\mu\text{M}$ ) was prepared. All points were measured in triplicate.

#### 2.1.2. ITC measurements

The ITC cell (1400  $\mu\text{L}$ ) was loaded with 75  $\mu\text{M}$  HSA solution or buffer alone. The titrations were carried out using an auto-syringe filled with the respective drug solution (4 mM DCF or 4 mM LIDO) with the stirring speed fixed at 307 rpm and temperature at 25  $^{\circ}\text{C}$ . Each experiment consisted of 35 consecutive injections (8  $\mu\text{L}$ ) of the drug solution for 16 s each, with a 360 s interval to allow complete equilibration. The control experiments were performed by titrating HSA solution with the buffer itself and by titration the net buffer with the drugs themselves to correct the data for the dilution heats of the HSA and drug, and for buffer mixing.

#### 2.1.3. CD measurements

The CD spectra of HSA (75  $\mu\text{M}$ ) incubated with the drug (0 – 500  $\mu\text{M}$ ), were recorded from 250 nm to 350 nm. All measurements were carried out at 25 $^{\circ}\text{C}$  using a cell with a 1 mm path length (200  $\mu\text{L}$ ). The data pitch was 0.1 nm with a 1 nm bandwidth at a scan speed of 50 nm/min. Each spectrum represents the average of four measurements.

#### 2.1.4. ED measurements

A new membrane was conditioned by rinsing with deionized water three times for 10 min and borate

buffer for 10 min. For the binding experiments, the series of samples containing a constant concentration of HSA (75  $\mu\text{M}$ ) and varying concentrations of drugs (100 - 800  $\mu\text{M}$ ) was prepared. The samples (500 $\mu\text{L}$ ) were pipetted into the donor chamber of the dialyzer and dialyzed against the acceptor chamber filled with neat buffer (500  $\mu\text{L}$ ). The dialysis was performed for 6 h at 25  $\pm$  1  $^{\circ}\text{C}$  under constant stirring at about 300 rpm on a MixMate<sup>®</sup> (Eppendorf, Germany).

The time to achieve the equilibrium state was determined experimentally. During its optimization, the samples containing a 100, 400 and 800  $\mu\text{M}$  solution of the drug were dialyzed against neat buffer and the drug concentration was analysed in both chambers of the dialyzer for increasing time periods.

## 2.2. *Theoretical Methodologies*

### 2.2.1. Data processing in CE-FA

Nonlinear regression was used to evaluate the binding parameters by substituting the experimental data according to the following equation:

$$r = \frac{[D_{\text{bound}}]}{[P_{\text{tot}}]} = \sum_{i=1}^m \frac{n_i \cdot K_{bi} \cdot [D_{\text{free}}]}{1 + K_{bi} \cdot [D_{\text{free}}]} \quad (1)$$

where  $r$  is the number of bound drug molecules per molecule of protein;  $[D_{\text{free}}]$ ,  $[D_{\text{bound}}]$ , and  $[P_{\text{tot}}]$  are the free drug, bound drug, and total protein concentrations, respectively;  $m$  is the number of identical independent binding classes;  $n_i$  is the number of sites of class  $i$ , and  $K_{bi}$  is the corresponding binding constant. The  $[D_{\text{free}}]$  was estimated from the plateau height (as it is illustrated in Fig. 1 - see below) and from the calibration curve obtained for the drug samples analysed in the absence of the protein. The  $[D_{\text{bound}}]$  was determined as the difference between the total  $[D_{\text{tot}}]$  and free  $[D_{\text{free}}]$  drug concentrations.

### 2.2.2. Data processing in ITC

For all the experiments, the heat of the binding reaction between HSA and the drug was obtained as the difference between the heat of reaction and the corresponding background heats of dilution/mixing. The experimental data were fitted to a binding model using a non-linear least-squares method with  $K_b$ ,  $n$  and  $H$  as adjustable parameters using the software Origin 7.0. The  $K_b$  was measured from the

slope of the binding curve and the inflection point characterized the number of binding sites.

### 2.2.3. Data processing in CD

The induced CD (ICD) (see Section 3.3 Circular dichroism) is defined as the CD of the HSA-drug mixture minus the CD of HSA alone at the same wavelength, and is expressed as ellipticity  $\Delta\theta$  in millidegrees (mdeg). The binding constant was determined by adjusting the experimental data using nonlinear regression (with MS Excel) according to the following equation [5]:

$$\Delta\theta = \frac{k}{2} \left( [P_{tot}] + [D_{tot}] + \frac{1}{K_{bi}} - \sqrt{\left( [P_{tot}] + [D_{tot}] + \frac{1}{K_{bi}} \right)^2 - 4[P_{tot}][D_{tot}]} \right) \quad (2)$$

where  $\Delta\theta$  is the ellipticity change due to complex formation (absolute value),  $[P_{tot}]$  and  $[D_{tot}]$  represent the total concentrations of HSA and drug, respectively, and  $k = 32982.1 \cdot \Delta\epsilon \cdot l$  ( $\Delta\epsilon$  is the extrinsic molar circular dichroic absorption coefficient of the drug bound to HSA in  $M^{-1} \text{ cm}^{-1}$  and  $l$  is the path length in cm).

### 2.2.4. Data processing in ED

Due to the fact that the free drug concentrations after equilibrium should be the same in both chambers of dialyzer, the total concentration of free drug was determined by measuring the free drug concentration in one of the part of the dialyzer and the obtained value was multiplied by two and used for further calculations.

## 3. Results and Analysis

Many different instrumental approaches have been developed over a time for investigating of protein-drug binding. Four of them were compared in this study. Two belong to so-called separative (CE-FA and ED) and other two belong to so-called non-separative method (ITC and CD).

As model drugs were chosen DCF and LIDO. DCF is a widely used nonsteroidal anti-inflammatory drug and it acts as a potent inhibitor of prostaglandin endoperoxide synthase, also known as cyclooxygenase. It is widely prescribed for the treatment of osteoarthritis, rheumatoid arthritis, ankylosing spondylitis and acute muscle pain

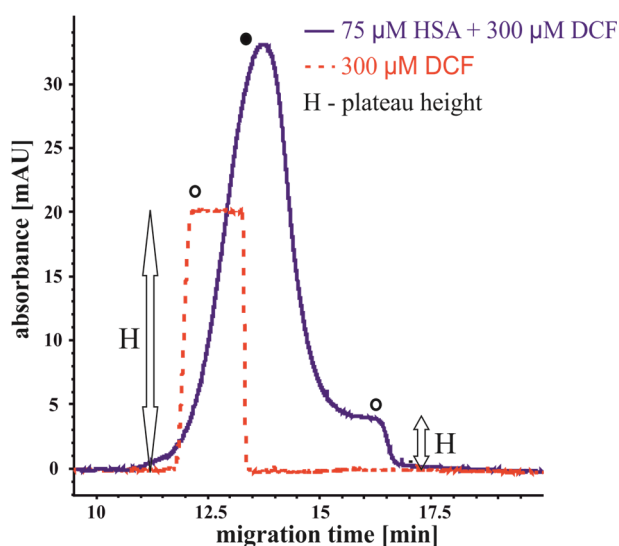


Figure 1. CE-FA electropherogram obtained by measuring drug standards and HSA-diclofenac mixture.

(○) indicates free diclofenac and (●) indicates HSA-diclofenac complex and free HSA.

conditions [6]. LIDO, a  $\text{Na}^+$  channel blocker, is used as a local anaesthetic and in the treatment of ventricular cardiac arrhythmias or cardiac arrest with ventricular fibrillation [7].

### 3.1. CE-FA

For studying biomolecular interactions can be used several CE-based experimental approaches. They have many common advantages for example no need for highly purified samples, measuring without immobilization or labeling, small sample consumption. In this study CE-FA approach was used due to its high sample throughput with possibility of automation. Typical electropherogram is illustrated in Fig. 1.

The determined  $K_b$  of the HSA-DCF interaction was  $(2.56 \pm 0.07) \cdot 10^4 \text{ L/mol}$  and  $n = 5.00 \pm 0.12$ ; the  $K_b$  of the HSA-LIDO interaction was  $(1.96 \pm 0.32) \cdot 10^3 \text{ L/mol}$  with  $n = 1.46 \pm 0.25$ .

### 3.2. ITC

This method is widely used to measure the affinity between two interacting molecules in aqueous solution. This method is useable for almost any interacting partners-nucleic acids, proteins, lipids, carbohydrates or organic compounds. Besides obtaining binding parameters this method provides also thermodynamic informatin.

The obtained thermodynamic parameters accompanying the binding of HSA-DCF show the negative entropy change ( $-125.48 \pm 7.20 \text{ J}\cdot\text{mol}^{-1}$ ) and negative enthalpy change ( $-60.75 \pm 2.01 \text{ kJ}\cdot\text{mol}^{-1}$ ). While the data on the HSA-DCF interaction indicated that the reaction was exothermic, the data on the HSA-LIDO interaction showed an endothermic reaction with a positive entropy change ( $86.04 \pm 7.79 \text{ J}\cdot\text{mol}^{-1}$ ) and positive enthalpy change ( $7.96 \pm 2.51 \text{ kJ}\cdot\text{mol}^{-1}$ ). The binding parameters for DCF to HSA were determined to be  $K_b = (1.22 \pm 0.09) \cdot 10^4 \text{ L/mol}$  and  $n = 2.00 \pm 0.11$ . The binding parameters for the second interaction pair, HSA and LIDO, were determined to be  $K_b = (1.28 \pm 0.24) \cdot 10^3 \text{ L/mol}$  and  $n = 1.39 \pm 0.27$ .

### 3.3. CD

CD method is usually used for studying the secondary structure of proteins and for investigation of reactions with respect to the chirality and stereochemistry. Measurement of the  $K_b$  is enabled by the phenomenon called induced Cotton effect – ICD. This effect appears in some cases within the absorption bands of achiral molecules which were otherwise CD inactive. The intensity of the ICD signal is proportional to the HSA-drug complex without interference from the free drug fraction. Obtained  $K_b$  value by CD method of the HSA-DCF was  $(7.91 \pm 0.12) \cdot 10^4 \text{ L/mol}$ . Unfortunately no ICD signal was detected for HSA-LIDO complex.

### 3.4. ED

This approach is considered as a reference method for the determining of binding parameters. Its principal advantage is physical simplicity, but the main drawback is time consumption, which can be eliminated by using multiwell plate format ED experiments.

The binding parameters were determined to be  $K_b = (3.38 \pm 0.11) \cdot 10^4 \text{ L/mol}$  and  $n = 5.08 \pm 0.07$  for the HSA-DCF pair and  $K_b = (2.05 \pm 0.48) \cdot 10^3 \text{ L/mol}$  and  $n = 2.13 \pm 0.17$  for the HSA-LIDO pair.

## 4. Discussion

In this study, binding parameters such as  $K_b$  and  $n$  for HSA-DCF and HSA-LIDO model systems were evaluated by CE-FA, ITC, CD and ED. The results

achieved and the literature values obtained by identical methods were compared. Values of  $K_b$  for HSA-DCF and HSA-LIDO pairs obtained by CE-FA, ITC and ED methods are comparable. A slightly higher result but still in the same concentration order of magnitude was measured for DCF using a CD method, unfortunately this trend could not be confirmed for the HSA-LIDO pair. The  $K_b$  values for the HSA-DCF pair are in good agreement with published results obtained using the same methods as in this study [8-10]. A slightly higher value was obtained by Vuignier et al. [11], however these authors used dissimilar conditions – a different BGE and BSA instead of HSA. Identical behaviour was observed for the HSA-LIDO pair, with a slightly higher  $K_b$  obtained by ED. This difference was probably due to using a different method for off line detection and a different buffer [11, 12].

Obtained values were also compared with  $K_b$  evaluated by different methods not used in this study and concentration values are in the same order of magnitude.

## 5. Conclusion

In this paper,  $K_b$  and  $n$  were measured by CE-FA, ITC, CD and ED to evaluate their potential for this pharmacologically important task. The integrating benefit of these methodologies is the possibility of measuring the interaction of binding partners in their native states without immobilization or labelling. Despite possibility of protein adsorption onto capillary wall the CE-FA method was selected as the best candidate for such a purpose, due to many advantages such as low sample consumption, automation and the possibility of studying both low- and high-affinity interactions. By the ITC method also thermodynamics parameters can be acquired, on the other hand large sample consumption and requirement for purified samples are limitations. Similarly, the CD-based method provides the structural information on the drug-protein complex, but it is preferable for study of the second structure because is suitable only for drugs affecting the protein structure. ED is considered a reference method for such studies, but it suffers from drawbacks, such as long equilibrium time, volume shifts associated with oncotic pressure and nonspecific adsorption.

## Acknowledgement

The research was supported by grant No. P206/12/G014 from the Czech Science Foundation. Part of the work was carried out with the instrumental support (ITC and CD) of Biomolecular Interactions and Crystallization Core Facility of CEITEC – Central European Institute of Technology.

## REFERENCES

- [1] Mendez-Lucio, O.; Medina-Franco, J.L.: The many roles of molecular complexity in drug discovery, *Drug Discov. Today*, 2017 **22**, 120-126 [10.1016/j.drudis.2016.08.009](https://doi.org/10.1016/j.drudis.2016.08.009)
- [2] Ruiz-Garcia, A.; Bermejo, M.; Moss, A.; Casabo, V.G.: Pharmacokinetics in drug discovery, *J. Pharm. Sci.* 2008 **97**, 654-690 [10.1002/jps.21009](https://doi.org/10.1002/jps.21009)
- [3] Fournier, T.; Medjoubi N., N.; Porquet, D.: Alpha-1-acid glycoprotein, *Biochim. Biophys. Acta-Protein Struct. Molec. Enzym* 2000 **1482**, 157-171 [10.1016/s0167-4838\(00\)00153-9](https://doi.org/10.1016/s0167-4838(00)00153-9)
- [4] Colmenarejo, G.: In silico prediction of drug-binding strengths to human serum albumin, *Med. Res. Rev.* 2003 **23**, 275-301 [10.1002/med.10039](https://doi.org/10.1002/med.10039)
- [5] Matei, I.; Ionescu, S.; Hillebrand, M.: Induced chirality of genistein upon binding to albumin: circular dichroism and tddft study, *Rev. Roum. Chim.* 2013 **58**, 409-413
- [6] Todd, P. A.; Sorkin, E. M.: Diclofenac sodium - a reappraisal of its pharmacodynamic and pharmacokinetic properties, and therapeutic efficacy, *Drugs* 1988 **35**, 244-285 [10.2165/00003495-198835030-00004](https://doi.org/10.2165/00003495-198835030-00004)
- [7] Bean, B. P.; Cohen, C. J.; Tsien, R. W.: Lidocaine block of cardiac sodium-channels, *J. Gen. Physiol.* 1983 **81**, 613-642 [10.1085/jgp.81.5.613](https://doi.org/10.1085/jgp.81.5.613)
- [8] Chen, J. B.; Zhou, X. F.; Zhang, Y. L.; Qian, Y. J.; Gao, H. P.: Interactions of acidic pharmaceuticals with human serum albumin: insights into the molecular toxicity of emerging pollutants, *Amino Acids* 2012 **43**, 1419-1429 [10.1007/s00726-012-1215-0](https://doi.org/10.1007/s00726-012-1215-0)
- [9] Indurthi, V. S. K.; Leclerc, E.; Vetter, S. W.: Calorimetric investigation of diclofenac drug binding to a panel of moderately glycosylated serum albumins, *Eur. J. Pharm. Sci.* 2014 **59**, 58-68 [10.1016/j.ejps.2014.04.002](https://doi.org/10.1016/j.ejps.2014.04.002)
- [10] Yamasaki, K.; Rahman, M. H.; Tsutsumi, Y.; Maruyama, T.; Ahmed, S.; Kragh-Hansen, U.; Otagiri, M.: Circular Dichroism Simulation Shows a Site-II-to-Site-I Displacement of Human Serum Albumin-Bound Diclofenac by Ibuprofen, *AAPS PharmSciTech* 2000 **1**, 45-54 [10.1208/pt010212](https://doi.org/10.1208/pt010212)
- [11] Vuignier, K.; Schappler, J.; Veuthey, J. L.; Carrupt, P. A.; Martel, S.: Improvement of a capillary electrophoresis/frontal analysis (CE/FA) method for determining binding constants: Discussion on relevant parameters, *J. Pharm. Biomed. Anal.* 2010 **53**, 1288-1297 [10.1016/j.jpba.2010.07.024](https://doi.org/10.1016/j.jpba.2010.07.024)
- [12] McNamara, P. J.; Slaughter, R. L.; Pieper, J. A.; Wyman, M. G.; Lalka, D.: Factors influencing serum-protein binding of lidocaine in humans, *Anesth. Analg.* 1981 **60**, 395-400

# PROSTATE-SPECIFIC ANTIGEN (PSA) IMMOBILIZATION FROM BIOLOGICAL SAMPLES FOR CAPILLARY ELECTROPHORESIS (CE) ANALYSIS

BALÁZS REIDER,<sup>1</sup> HAJNALKA JANKOVICS,<sup>1</sup> GÁBOR JÁRVÁS,<sup>1,2</sup> FERENC VONDERVISZT,<sup>1</sup>  
AND ANDRÁS GUTTMAN<sup>1,2,3</sup>

1 Research Institute for Biomolecular and Chemical Engineering, University of Pannonia, Veszprem, Hungary

2 MTA-PE Translational Glycomics Research Group, Research Institute for Biomolecular and Chemical Engineering, University of Pannonia, Veszprem, Hungary

3 Horváth Laboratory of Bioseparation Sciences, University of Debrecen, Hungary

## Abstract

Prostate cancer is one of the most commonly diagnosed cancer in men worldwide. Serum prostate-specific antigen (PSA) is a well-known biomarker for this disease, thus it is frequently used for prostate cancer screening or monitoring disease progression. However the lack of selectivity and sensitivity of this method often leads to over-diagnosis and over-treatment, therefore the discovery of new biomarkers is of high importance. It is reported earlier that prostate cancer - like many other tumor diseases - modify the glycan profile of certain proteins. Alteration of the PSA glycoform can be detected using highly sensitive analytical methods. Laser induced fluorescent detected capillary electrophoresis have been proved to be suitable for this task, although the limit of detection is restrictive for most of the biological samples. Other glycoproteins could interfere with the analysis as well, thus the capture and preconcentration of PSA from the samples are essential. Monoclonal antibodies (mAbs) are widely used for PSA immobilization due to their strong affinity and high selectivity. On the other hand, mAbs contain glycan structures which can cause contamination in the samples. Furthermore, their price is very high and their sensitivity can require special conditions.

In this study a new method is presented for PSA capture from low-concentration samples among others, semen, urine or blood. Single domain antibodies (sdAb) are smaller in size and more stable than mAbs and have no any glycosylation sites. They can be produced in bacteria with recombinant techniques, which makes them cheaper, furthermore various linker tags like His-tag or GST-tag can be attached to them. Amino acid sequences of sdAb (clone N7 and C9) were taken from "Single Domain Antibodies Recognizing Different PSA Isoforms (doi: 10.1074/jbc.M409292200)" and were synthesized by GenScript. The genes were amplified by PCR, digested with NdeI and XhoI restriction endonucleases and after purification on agarose gel, ligated into pET23b vector which fuses the DNA sequence of a C-terminal 6-histidine tag to the aPSA genes. SHuffle T7 Express Competent E. coli bacteria (New England Biolabs) were cloned by the resulting plasmids to ensure correct disulphide bond formation and protein folding.

Various sample volume to antibody concentration ratios were tested in the preliminary experiments using magnetic particles. SiMAG-IDA/Nickel nickel-coated magnetic microbeads (provided by Chemicell) were applied. Later, packed pipette tips (Ni-IMAC PhyTipPhyNexus, San Jose, CA) were used. By automatic pipetting the C9 antibodies proved to be able to be immobilized to the columns. Switching the antibody vials to sample vials the capture of PSA from the samples could be actualized. As the next step PSA could be gained from the column by denaturing the proteins. Consequently, the denatured PSA can easily be connected to the standard glycan sample preparations for CE analysis. The method was applied with promising results on standard PSA from human semen (SigmaAldrich, St. Louis,



MI) solution in PBS buffer and formalin fixed standard PSA samples as well. The further efforts are aiming to apply this protocol on urine samples and successfully connect it to CE glycan analysis.

### **Acknowledgment**

The authors gratefully acknowledge the support of the Momentum (Lendulet) grant # 97101 of the Hungarian Academy of Sciences (MTA-PE Translational Glycomics) and the National Research, Development and Innovation Office (NKFIH) (K 116263) grants of the Hungarian Government. This work was also supported by the BIONANO\_GINOP-2.3.2-15-2016-00017 project.

# IN FLIGHT ANALYSIS OF ASTRONAUT'S SERUM IMMUNOGLOBULINS DURING DEEP SPACE MISSIONS; THE NEXT LEAP IN CAPILLARY ELECTROPHORESIS

MÁTE SZARKA<sup>1</sup>, SZABOLCS SZILASI<sup>2</sup>, ANDRÁS GUTTMAN<sup>1</sup>

1 Horváth Laboratory of Bioseparation Sciences, University of Debrecen, Hungary

2 Institute for Nuclear Research, Hungarian Academy of Sciences, Debrecen, Hungary

## Abstract

On a roundtrip (~360 days) to Mars, astronauts would be exposed to approximately 0.66 Sv (1) radiation dose, mainly due to Galactic Cosmic Rays (GCR) and Solar Energetic Particles (SEP). This amount of exposure exceeds the lifetime limits here on Earth according to RAD measurements (1). Previous studies have shown that after extensive radiation exposure, inflammatory proteins such as cytokines are induced in the human body. In general, radiation modulates the hematopoietic system (2). Under the exposure to strongly ionizing radiation, serum glycoprotein (IgG and IgM) levels in the blood initially drastically decrease even to hypogammaglobulinemic levels but in a long term, a regenerative hypercompensation is observed (3). Specific glycosylation patterns of these immunoglobulins, especially IgG have already been associated with various ailments such as chronic inflammation, autoimmune diseases and malignant transformation just to mention a few. Moreover glycan based biomarkers indicating biological age (4) are of significant interest in the monitoring of age-related diseases known to accelerate in space. The focus of our work is to profile the IgG glycans after medium-energy proton irradiation to reveal the resulting possible glycosylation profile changes. Structural elucidation will be accomplished by our small and lightweight image analysis based LED induced capillary electrophoresis system to decipher the effects and threats of SEP on possible physiological changes of future astronauts during long duration missions beyond the shielding environment of Low Earth Orbit (LEO).

## References:

- [1] C. Zeitlin et al. *Science*, 2013, 340, 1080-1084
- [2] M. Deepa, et al., *Life Sciences in Space Research*, 2016, 9, 19–47
- [3] J. Andrlíková J, Wagner V, Pálek V; *Strahlentherapie*, 1975, 149, 212-8.
- [4] V. Vanhooren et al. *Experimental Gerontology*, 2010, 45, 738-43.

# MICROFLUIDIC CELL COUNTING DEVICE BASED ON RESISTIVE PULSE SENSING

TOMAS VACLAVEK<sup>1,2\*</sup>, JANA KRENKOVA<sup>1</sup>, AND FRANTISEK FORET<sup>1</sup>

<sup>1</sup> Department of Bioanalytical Instrumentation, Institute of Analytical Chemistry of the Czech Academy of Sciences, Veveří 967/97, Brno, 60200, CZECH REPUBLIC

<sup>2</sup> Department of Biochemistry, Faculty of Science, Masaryk University, Kamenice 753/5, Brno, 625 00, CZECH REPUBLIC

**Abstract** – In this lecture we present the microfabrication procedure of a particle counting device based on resistive pulse sensing in a microfluidic channel. This microfluidic device was entirely made of borosilicate glass by means of photolithography and glass etching. Its performance was confirmed by measuring samples of polystyrene particles and various types of biological cells.

**Keywords:** microfluidic, cell, counting, resistive, pulse

## 1. Introduction

Microfluidic devices show a great potential for analysis of cell populations as a single microfluidic chip can be designed to perform multiple operations, e.g. lysis and staining, allowing us to analyze injected cells in small groups or one by one. In such experiment it is important to precisely control the number of entering cells. Therefore integration of a cell counting unit represents an important step in development of a microfluidic device for cellular analysis.

Cells flowing through the microchannel can be registered visually using a camera and software image analysis. This approach requires a fast camera with an objective and software algorithm to recognize a cell moving through the microchannel, distinguish it from the background and other cells in case of multiple cells moving in a group.

Resistive pulse sensing (RPS) is an alternative approach that is based on sensing disruptions in a current flow caused by cells passing through the narrow section, often called the sensing gate. The greatest electric current density is at the sensing gate where volume of conductive electrolyte is the smallest thus the overall resistance of a microchannel is given by this narrowest section. When a non-conductive particle enters this section conductivity drops proportionally to the volume of the particle. This phenomenon also allows the system to distinguish a particle size according to the height of resistive pulse. In terms of microfluidic cellular analysis this ability could be used as the first characterization of cells or as a sorting criterion.

## 2. Experimental

### 2.1. Microfabrication procedure

Design of a microfluidic device was created in AutoCAD (AutoDesk, USA). At first a replication mask was created by exposing this design using a high-resolution direct laser-writing system  $\mu$ PG 101 Micro-pattern Generator (Heidelberg Instruments, Germany) on commercially available photomask blank substrates (Nanofilm, USA). This replication mask was then used to transfer the design on borosilicate glass wafers coated with 60 nm of chromium and 70 nm of gold as masking metal layers and S1805 positive photoresist (Dow Electronic Materials, USA). These masking metal layers were sputtered using a Sputter-coater Q300TD (Quorum, England). After development of photoresist and etching of masking metal layers the borosilicate wafer was etched in a diluted mixture of sulfuric and hydrofluoric acid at 45°C for 5 minutes to reach the depth of approximately 12  $\mu$ m. Then the wafer was washed in deionized water to stop etching, acetone to remove the photoresist and piranha solution (sulfuric acid : hydrogen peroxide, 3:1) to clean the surface for the second metal sputtering process. Only the main channel and the sensing gate were again coated with 60 nm of chromium and 70 nm of gold to protect these areas from further etching. Afterwards the inlet channels were etched to the depth of 250  $\mu$ m so the platinum electrodes and fused silica capillaries could be smoothly inserted in. Two etched wafers were aligned and thermally bonded in a muffle furnace

---

\*Correspondence: vaclavek@iach.cz

at 620°C for 10 hours so the resulting microchannel had a circular cross section.

In order to tightly seal the inlets for platinum wire electrodes and fused silica capillaries a plastic frame was designed in SketchUp (Google, USA) and printed out using the 3D printer Easy3DMaker (3D Factories, Germany) based on fused deposition modelling technology.

### 2.2. Resistive pulse sensing

The microfluidic device was placed on a SVM 340 synchronized video microscope (LabSmith, USA) to have a visual control over cells running through the sensing gate.

A colibrick A/D converter and Clarity Chromatography Data Station (Data Apex, Czech Republic) were used to monitor resistive pulses by measuring voltage at the output of the conductometric detector (Villa Labeco, Slovakia). Resistive pulse signals were recorded using a data acquisition rate of 100, 200 and 400 Hz.

### 2.3. Samples

At first the initial experiments were performed with monodisperse 10 µm polystyrene beads (Sigma-Aldrich, Germany), which were chosen as a non-conductive particle size standard, suspended in the PBS buffer. In experiments with biological cells we observed excessive clumping of cells especially of *Saccharomyces cerevisiae* cells, therefore it was necessary to add 0.1 mM EDTA into PBS buffer to suppress this cell to cell adhesion. Further we performed experiments with mouse osteoblasts MC3T3-E1 and human prostate cancer PC3 cells.

MC3T3-E1 mouse osteoblasts and PC3 human prostate cancer cells were collected and centrifuged at 600 G. Growing medium was removed and the cell pellet was resuspended in PBS. Samples were then again centrifuged at 600 G and resuspended in PBS with addition of 0.1 mM EDTA for experiments.

*Saccharomyces cerevisiae* cells were purchased as dry pellets and were just resuspended in PBS buffer containing 0.1 mM EDTA.

## 3. Results and Discussion

### 3.1. Microfabrication

The two-step etching process resulted in slightly over-etched edges in microchannels which had been etched in the first step and re-coated with 60 nm of chromium and 70 nm of gold. This re-coating was an insufficient protection from further etching in the second step, especially at the edges of microchannels. Chromium and gold is the best combination of metals for protection in glass etching. In our laboratory it was proven that 60 nm of chromium as a first layer with 70 nm of gold as a

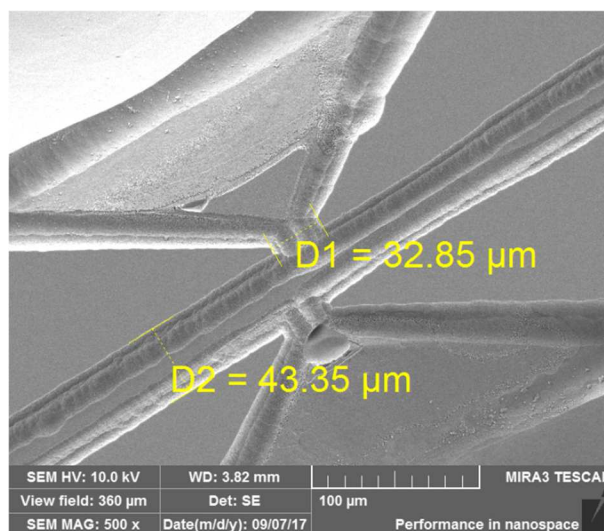


Figure 1. Scanning electron microscope image of the sensing gate shows the diameter of microchannel as D2.

second layer was sufficient protection for flat surfaces even in deep etching (reaching depths of approx. 250 µm). However in this case it was insufficient to protect the edges of microchannels etched in the previous step, probably because the coating process is not equally effective at the edges as at the flat surface even though the sputtering machine had a rotating stage.

The resulting diameter of a microchannel was 43 µm according to the scanning electron microscope image in Fig. 1.

### 3.2. Resistive pulse sensing

As particles and especially biological cells may form coagulates it was crucial to identify single particle peaks by observing particles on a microscope at a reduced flow rate. The digital representation of the actual voltage was recorded in

a form of a chromatogram (as Clarity software was designed for chromatography stations) and in our experimental conditions (low flow rate) it was sufficient to set the data acquisition frequency to 100 Hz. Clarity chromatograms were exported into MS Excel and as a defining criterion was chosen the peak height, because it refers to particle size (more specifically to the volume ratio of a particle to sensing gate). Although in chromatography is more commonly used the peak area, in terms of resistive pulses the base of a peak refers to the time of particle passage, which is actually dependent on the velocity of flow. Therefore the peak height has higher significance.

To evaluate an average resistive pulse signal of different particle samples we selected 10 single particle peaks and calculated the height of every peak by subtracting the averaged value of baseline (averaged value of baseline was calculated by averaging 40 data points surrounding each peak).

Resistive pulse signals for polystyrene particles and various types of biological cells are summarized in *Tab. 1*. It is worth to note that in case of monodisperse polystyrene particles the slight variation in peak heights is caused by differences in particles trajectories. The magnitude of disruptions in electric current flow at the sensing gate slightly differs when these disruptions occur in the middle of a microchannel or closer to the microchannel walls [1].

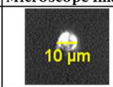
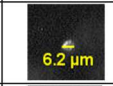
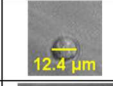
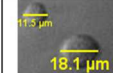
#### 4. Conclusion

The microfluidic device presented here is compatible with fused silica capillaries, therefore it facilitates its combination with electromigration methods in a capillary format. More importantly, as opposed to microfluidic devices based on PDMS, it can be repeatedly disassembled, cleaned using harsh cleaning agents (eq. piranha, chromic acid) and reassembled to achieve full performance after clogging of microchannels. This device has a potential to be a particle counting module in a tailored flow system where is required to monitor the number of entering particles into subsequent microfluidic operations or electrophoretic separations.

#### Acknowledgement

The research was supported by the Grant Agency of the Czech Republic (16-09283Y, P206/12/G014) and the institutional research plan (RVO: 68081715) of the Institute of Analytical Chemistry of the Czech Academy of Sciences.

*Table 1.* Average resistive pulse heights measured for polystyrene particles and various types of biological cells.

Particle Type	Microscope Image	Average Pulse Height [mV]	SD [mV]
10 um polystyrene beads		0.181	0.011
<i>Saccharomyces cerevisiae</i>		0.076	0.012
Mouse osteoblasts MC3T3-E1		0.670	0.179
Human prostate cancer PC3		1.744	0.843

#### REFERENCES

- [1] Weatherall, E.; Hauer, P.; Vogel, R.; Willmott, G.R.: Pulse Size Distributions in Tunable Resistive Pulse Sensing, *Anal. Chem.*, 2016 **88**, 8648–8656 DOI: 10.1021/acs.analchem.6b01818

# Abstracts of poster presentations

## P1 IDENTIFICATION OF O-GLYCOSYLATED (N-TERMINAL) PRO-B-TYPE NATRIURETIC PEPTIDE FORMS IN BLOOD PLASMA OF PATIENTS WITH SEVERE HEART FAILURE

BENNO AMPLATZ,<sup>1</sup> BERNHARD HALFINGER,<sup>1</sup> ANGELIKA HAMMERER-LERCHER,<sup>2</sup> BETTINA SARG,<sup>1</sup> LEOPOLD KREMSER,<sup>1</sup> AND HERBERT H. LINDNER<sup>1\*</sup>

<sup>1</sup> Division of Clinical Biochemistry, Innsbruck Medical University, Innsbruck, A-6020, AUSTRIA

<sup>2</sup> Institute of Laboratory Medicine, Kantonspital Aarau AG, Aarau, Switzerland.

Abstract – We identified nine distinct glycosylation sites on circulating (NT-) proBNP in heart failure patients using immunoaffinity purification, sequential exoglycosidase treatment for glycan trimming and high-resolution nano-flow liquid chromatography electrospray multistage mass spectrometry. Our results directly demonstrate a rather complex and interindividually different distribution of the endogenously circulating glycoforms. This information may also have an impact on commercial immunoassays applying antibodies specific for the central region of (NT-) proBNP, which detect mostly nonglycosylated forms.

**Keywords:** brain natriuretic peptide (BNP), O-glycopeptides, Heart Failure.

### 1. Introduction

N-terminal pro-B-type natriuretic peptide (NT-proBNP) and its C-terminal physiologically active counterpart, B-type natriuretic peptide (BNP), have become well-accepted heart failure (HF) biomarkers and were implemented into the guidelines for HF-management more than 10 years ago. Both forms, as well as their unprocessed precursor proBNP circulate as various N- and C-terminally truncated and O-glycosylated forms in severely ill HF patients.

As the proBNP : BNP ratio was reported to increase in pathophysiological conditions such as ventricular overload and O-glycosylation of proBNP impedes its procession, understanding the structural attributes and molecular heterogeneity of the different natriuretic peptide B forms displays a critical prerequisite for improved design of assays as well as their clinical application in the diverse settings of HF.

In this study we set out to define the specific O-glycosylation sites of human endogenous (NT-) proBNP in HF patients. We also demonstrate a variable distribution of glycoforms in different patients.

### 2. Experimental

#### SAMPLE PREPARATION

Heparinized plasma samples with concentrations above 10,000 pg/ml were purified by Immunopurification using either a biotin-conjugated polyclonal antibody (pAb) directed against amino acids (aa) 1–21 or biotin-conjugated F(ab')<sub>2</sub> fragments of a mAb specific to aa 42–46 of (NT-) proBNP bound to streptavidin-coated magnetic microparticles (Roche Diagnostics). Eluates were partially deglycosylated with an exoglycosidase cocktail and subsequently proteolytically digested with trypsin or endoproteinase Glu-C or by a combination of both enzymes.

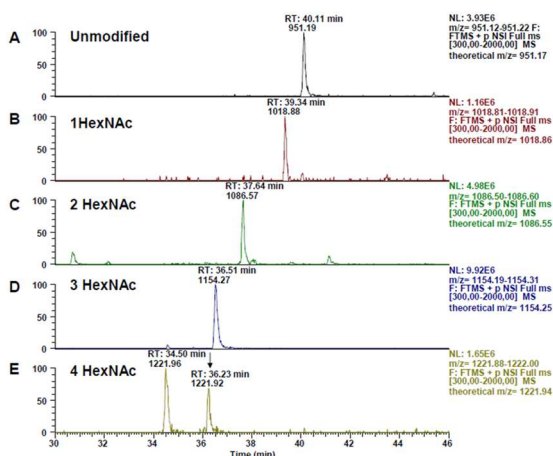
#### MASS SPECTROMETRIC ANALYSIS OF IMMUNOPURIFIED ENDOGENOUS (NT-) proBNP

Proteolytically digested samples were analyzed by nanoflow liquid chromatography electrospray ionization multistage mass spectrometry (nano-LC ESI-MSn) on an LTQ Orbitrap XL (Thermo Fisher Scientific).

\*Correspondence: [Herbert.Lindner@i-med.ac.at](mailto:Herbert.Lindner@i-med.ac.at)

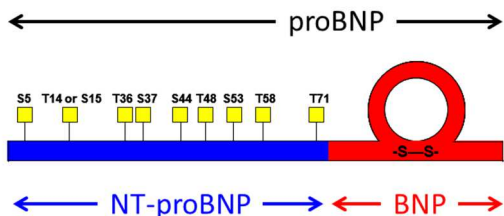
### 3. Results and Analysis

In a nano-LC ESI-MS3 analysis of peptide 1-13



**Figure 1.** Nano-LC ESI-MS of a tryptic digest of an IP-enriched plasma fraction pooled from 9 patients after limited exoglycosidase treatment. (A–E): XICs of glycoforms of the peptide 28–52. Peaks represent the monoisotopic  $m/z$  ratios ( $z=3+$ ) with 50 ppm mass tolerance. The arrow in (E) indicates an interfering peptide. RT: retention time;

(generated by an endoproteinase Glu-C digest) a new glycosylation site could be identified: Out of the three possible sites, the monoglycosylated form was pinpointed to Ser5.



**Figure 2.** Scheme of identified glycosylation sites in (NT-)proBNP.

Glycosylated positions are indicated as yellow boxes.

On peptide 14–21 (generated by a trypsin / Glu-C codigest), which carries two possible glycosylation sites, either Thr14 or Ser15 was found to be substoichiometrically glycosylated. Neither site has been described to be glycosylated previously as well.

The tryptic peptide 28–52, which has 5 potential glycosylation sites, was found in a nonglycosylated form, but also mono-, di-, tri-, and tetraglycosylated. Data evaluation proved that Ser29 is not significantly glycosylated, whereas

Thr36, Ser37, Ser44, and Thr48 are modified by glycans. These glycoforms were well resolved in nanoflow reversed-phase chromatography according to the number of attached glycans (Fig. 1 A–E). Interestingly, relative amounts of glycoforms varied between patients.

Finally, analysis of tryptic peptides 55-66 and 66–73 assigned a further glycosylation site to Thr58 and confirmed the glycosylation site at Thr71. For an overview of all glycosylation sites identified on (NT-)proBNP, see Figure 2.

### 4. Conclusion

In this study, we characterized nine specific O-glycosylation sites on human (NT-) proBNP circulating in plasma of patients with severe HF. Most strikingly, all peptides characterized coexist also in an unmodified form and relative amounts of proteolytic glycoforms varied among patients.

For a more elaborate version of this study, we encourage readers to see the full scientific publication on which this abstract for CECE 2017 (14th International Interdisciplinary Meeting on Bioanalysis) in Veszprém, Hungary is based [1].

### Acknowledgement

Research Funding: Research grant from Roche Diagnostics GmbH, Penzberg, Germany (D150820-011-012).

### REFERENCES

[1] Halfinger B., Hammerer-Lercher A., Amplatz B., Sarg B., Kremser L., Lindner H.H.: Unraveling the Molecular Complexity of O-Glycosylated Endogenous (N-Terminal) pro-B-Type Natriuretic Peptide Forms in Blood Plasma of Patients with Severe Heart Failure *Clin. Chem.* 2016 63(1) 359-368 DOI:10.1373/clinchem.2016.265397

## P2 CAPILLARY ISOTACHOPHORESIS DETERMINATION OF TRACE OXIDIZED GLUTATHIONE IN BLOOD

RÓBERT BODOR,<sup>1\*</sup> ANDREA NEČASOVÁ,<sup>2\*</sup> ALENA PECHOVÁ<sup>2</sup> AND MARIÁN MASÁR<sup>1</sup>

<sup>1</sup> Department of Analytical Chemistry, Faculty of Natural Sciences, Comenius University in Bratislava, Ilkovičova 6, Bratislava, SK-84215, SLOVAK REPUBLIC

<sup>2</sup> Department of Animal Protection, Welfare and Behaviour, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Palackého tř. 1/3, Brno, CZ-61242, CZECH REPUBLIC

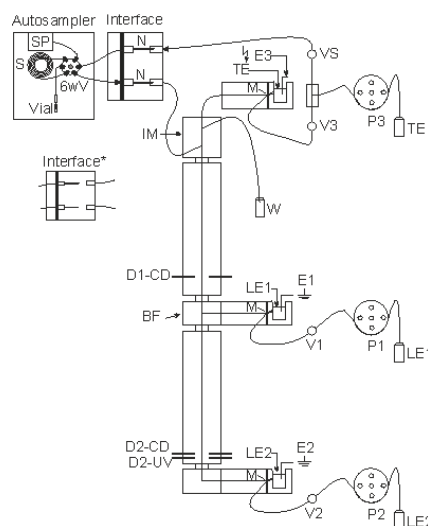
**Abstract** – A capillary isotachopheresis (CITP) method performed in a column-coupling equipment has been developed for the simultaneous determination of glutathione (GSH) and glutathione disulfide (GSSG) in blood samples. Determination of GSSG and GSH in biological samples is important because of their roles in oxidative stress. Different concentrations of leading ion in the coupled columns (concentration cascade) and high volume (37  $\mu$ L) of injected sample allowed the determination of GSSG in the concentration range 2-25  $\mu$ mol/L. Sample preparation involved reaction between iodoacetate and GSH under alkaline conditions in order to avoid oxidation of GSH to GSSG. This step eliminated the main source of systematic errors in the GSSG determination. Linear relationship ( $R^2=0.9969$ ) between the zone length of S-(Carboxymethyl) glutathione (product of GSH and iodoacetate) and the concentration of GSH (40-120  $\mu$ mol/L) was obtained. The method was applied to the analysis of ten times diluted bovine blood samples with satisfactory results.

**Keywords:** glutathione, glutathione disulfide, isotachopheresis, bovine blood

### 1. Introduction

Glutathione (GSH), a thiol-containing tripeptide, plays an important role in the antioxidant system of eukaryotic cells [1]. Upon oxidation, GSH is transformed to glutathione disulfide (GSSG) [1]. The concentration levels of GSH and GSSG and their molar ratio are indicators of cell functionality as well as oxidative stress [2]. Different aspects of the determination of GSH and GSSG in biological samples, including sample pretreatment, was recently reviewed [3-5]. The main problems are related to the non-enzymatic GSH autoxidation at  $\text{pH} > 7$ , enzymatic conversion of GSH, a need for the removal of proteins prior to the analysis, blocking of free thiol groups, reduction of disulfides and derivatization of thiol group [3, 6]. Among a wide variety of analytical methods, capillary electromigration methods, mainly CE coupled with different detectors (UV absorbance [7-10], fluorescence [11] and laser-induced fluorescence [12, 13], electrochemical [14, 15] and mass spectrometry [16]) have been used for the determination of glutathione [5, 17].

The aim of this work was to develop a capillary isotachopheresis (CITP) method with minimal sample pretreatment for simultaneous determination of GSSG and GSH in bovine blood



**Figure 1.** A scheme of the electrophoresis system. Autosampler: S – sample loop, SP – syringe pump, 6wV – 6-way valve. Interface: during the injection the septums are pierced by the needles (N). Interface\*: during the separation the autosampler is disconnected from the analyzer. Separation and electrolyte unit: V1-V4 – pinch valves, E1-E3 – driving electrodes, P1-P3 – peristaltic pumps, IM – injection module, W – waste, D1-CD, D2-CD, D2-UV – contactless conductivity and absorbance detection cells, M – membrane, BF – bifurcation, TE, LE1, LE2 – terminating and leading electrolytes.

\*Correspondence: [robert.bodor@uniba.sk](mailto:robert.bodor@uniba.sk)



Table 1. Electrolyte systems

Electrolyte	Parameter	ES1	ES2
LE1	Leading ion	Chloride	
	Concentration [mmol/L]	10	10
	Counter ion	6-Aminocaproate	
	Concentration [mmol/L]	12	12
	EOF suppressor	Methylhydroxyethylcellulose	
	Concentration [% w/V]	0.1	0.1
	pH	3.4	3.4
LE2	Leading ion	Chloride	
	Concentration [mmol/L]	5	2
	Counter ion	6-Aminocaproate	
	Concentration [mmol/L]	6	2.4
	EOF suppressor	Methylhydroxyethylcellulose	
	Concentration [% w/V]	0.1	0.1
	pH	3.8	3.9
TE	Terminating ion	Caproate	
	Concentration [mmol/L]	20	20
	Counter ion	6-Aminocaproate	
	Concentration [mmol/L]	15	15
	EOF suppressor	Methylhydroxyethylcellulose	
	Concentration [% w/V]	0.1	0.1
	pH	4.7	4.7

samples. Determination of trace analytes in regular CITP, when the quantitation is based on the zone length measurement, is not very common. In this work, two key problems were solved: (1) lowering the limit of quantitation of the CITP method, and (2) stabilization of GSH without protein precipitation for accurate GSSG determination.

## 2. Experimental

An EA 202A analyzer (Villa-Labeco, Spišská Nová Ves, Slovak Republic) was used for CITP separations. This fully automated CE system was equipped with two columns. In the first column, a fluoroplastic capillary tube with ID of 800  $\mu\text{m}$  and contactless conductivity detection were used. In the second column, a fused silica capillary with ID of 300  $\mu\text{m}$ , contactless conductivity and UV photometric detectors were used.

The CITP separations were performed using electrolyte systems shown in Table 1 at constant driving current, and in anionic mode (electrode E3 in Fig. 1 used as a cathode). At the beginning and at the end of the day, the separation and electrolyte units as well as sample loop of the autosampler were rinsed with deionized water using built-in peristaltic pumps. Between analyses a relatively short rinsing procedure (ca. 1 min) with electrolyte solutions was used.

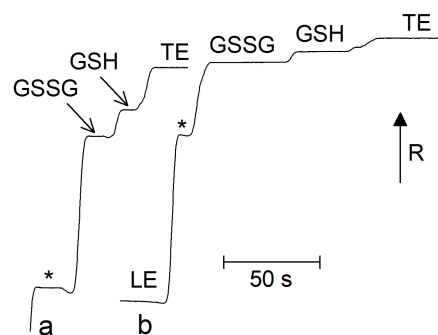


Figure 2. Isotachopherograms from the separations of GSSG and GSH performed in the electrolyte system ES1 (a) and ES2 (b). The isotachopherograms were recorded by D2-CD (Fig. 1). Concentration of GSSG and GSH in the injected sample was 25  $\mu\text{mol/L}$ .

### 2.1. Samples

The bovine blood samples were collected using PVC taking set with integrated needle HEMOS (Gama Group, České Budějovice, Czech Republic), and immediately transferred to the test tube with K<sub>3</sub>EDTA. The samples after dilution, required for hemolysis, were filtered prior to the analysis using syringe filter with glass fiber membrane with pore size of 1  $\mu\text{m}$ . During the dilution step a thiol-masking agent and NaOH was added to each sample.

## 3. Results and Discussion

### 3.1. Separation conditions

The combination of two columns with different IDs, employing a column switching technology, is beneficial for the CITP determination of analytes present in the multicomponent sample at low concentrations and/or at different concentration levels. The first (wider) capillary has allowed the separation of sample constituents loaded on the column at relatively high volume (37  $\mu\text{L}$ ). Typical macroconstituents, e.g., chloride and EDTA migrated out of the separation path through

bifurcation block, and by this way they were removed from the separation compartment. During this stage of the separation the driving current flows between electrodes E3 – E1 (Fig. 1).

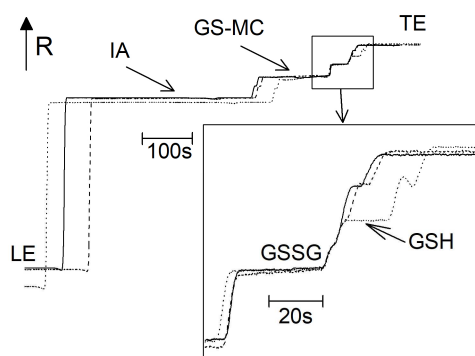


Figure 3. Isotachopherograms from the separations of reaction mixture performed in the electrolyte system ES2. The isotachopherograms were recorded by D2-CD. Mixture contained 15  $\mu\text{mol/L}$  GSSG, 80  $\mu\text{mol/L}$  GSH, 2 mmol/L IA, 4 mmol/L NaOH, 10 % TE. The sample was injected at 5 min (dot), 15 min (dash) and 20 min (solid) after mixing of the reagents.

Very short ITP zones of analytes created in the first column were not sufficient for their determination. By switching the direction of the driving current through both columns (by connection of electrodes E3 and E2, Fig. 1), the separated constituents were transferred to the second column. An appropriate timing of the switching of the current has been achieved using signal from D1-CD (Fig. 1).

In the second (narrower) capillary the ITP zones of analytes were prolonged. In addition, due to the low GSSG concentration in the blood samples (maximally tens of  $\mu\text{mol/L}$ ), a further extension of its ITP zone length was done by the use of lower concentration of leading ions (ES2, Table 1) in the second column (Fig. 2). Higher sensitivity achieved

Table 2. Parameters of regression equations

Analyte	Range [ $\mu\text{mol/L}$ ]	a [s.L/ $\mu\text{mol}$ ]	b [s]	$R^2$
GSSG <sup>1</sup>	5-50	0.67	1.45	0.9997
GSSG <sup>2</sup>	2-25	1.66	2.01	0.9992
GSH <sup>1</sup>	10-50	0.48	0.25	0.9999
GSH <sup>2</sup>	10-50	1.24	0.60	0.9994
GSH (GS-MC) <sup>2</sup>	40-120	1.98	-6.60	0.9969

<sup>1</sup> Electrolyte system ES1, and <sup>2</sup> ES2 used for data evaluation. Regression equation:  $Y = aX + b$ .

in electrolyte system ES2 is also evident from the parameters of regression equations for the analytes (Tab. 2).

### 3.2. Stabilization of glutathione

The stability of GSH and its oxidation to GSSG during the period between the sample collection and the analysis is the main source of systematic errors. In bovine blood sample we determined much higher concentration of GSSG and lower concentration of GSH than expected values.

To avoid this problem, we used thiol-masking agent, iodoacetic acid (IA) [18]. The substitution reaction between IA and GSH formed S-(Carboxymethyl) glutathione (GS-MC).

Under the ITP separation conditions used, GS-MC migrated in front of GSSG (Fig. 3). The optimal conditions for the reaction between IA and GSH was determined by the ITP separations of reaction

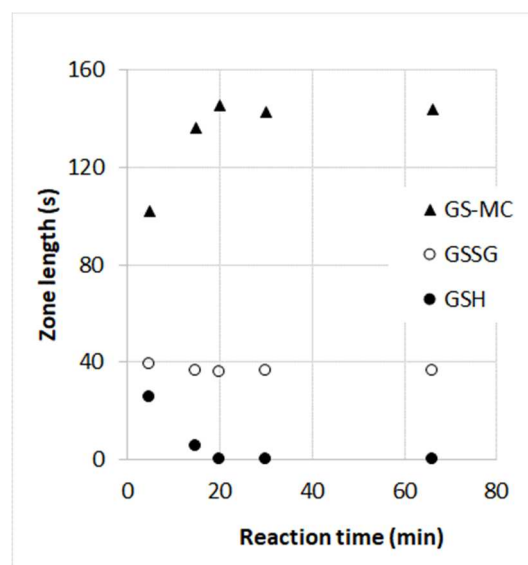


Figure 4. Dependence of ITP zone length on the reaction time of the mixture containing 15  $\mu\text{mol/L}$  GSSG, 80  $\mu\text{mol/L}$  GSH, 2 mmol/L IA, 4 mmol/L NaOH, 10 % TE.

mixtures in different times from the mixing of reagents. These experiments were carried out with both, model and real samples. At neutral and slightly alkaline pH the reaction was very slow. An excess of IA (2 mmol/L) and presence of NaOH (4 mmol/L) in the reaction mixture leads to the fast (less than 20 min) and quantitative conversion of GSH to GS-MC (Fig. 3 and Fig. 4) without increasing of the GSSG concentration.

For quantitative analysis, IA and NaOH was added to the bovine blood sample immediately after its collection (during sample dilution step required for hemolysis). Ten times diluted blood samples were directly analyzed after their filtration through a syringe filter (1  $\mu\text{m}$  pore-size). Determined concentrations of GSSG and GSH in the diluted blood sample were 4.4  $\mu\text{mol/L}$  and 63.4  $\mu\text{mol/L}$ , respectively. The average concentrations calculated from eight repetitive measurements of identical sample were in a good agreement with those determined by enzymatic method. The precision of the method, expressed by the RSD values of determined concentration of GSSG and GSH was 10.3% and 4.4%, respectively.

#### 4. Conclusion

The developed capillary isotachopheretic method allows a sensitive and simultaneous determination of GSH and GSSG in whole bovine blood samples. Simple and rapid preparation of blood samples, involving only dilution, masking of thiol group in GSH and filtration, increases the accuracy of the GSSG determination. No adverse effects of the proteins present in the real blood samples on the separation efficiency or detector response was observed. It can be assumed that this method is also suitable for the analysis of the blood samples of other mammals.

#### Acknowledgement

The research was supported by the Slovak Research and Development Agency (APVV-0259-12), and the Scientific Grant Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic and the Slovak Academy of Sciences (VEGA 1/0342/15). One of us (A.N.) thanks for mobility grant 2017-FVHE-11.

#### REFERENCES

- [1] Meister, A.; Anderson, M.E.: Glutathione, *Annu. Rev. Biochem.*, 1983 **52**(1), 711-760 10.1146/annurev.bi.52.070183.003431
- [2] Schulz, J.B.; Lindenau, J.; Seyfried, J.; Dichgans, J.: Glutathione, oxidative stress and neurodegeneration, *Eur. J. Biochem.*, 2000 **267**(16), 4904-4911 10.1046/j.1432-1327.2000.01595.x
- [3] Monostori, P.; Wittmann, G.; Karg, E.; Túri, S.: Determination of glutathione and glutathione disulfide in biological samples: An in-depth review, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.*, 2009 **877**(28), 3331-3346 10.1016/j.jchromb.2009.06.016
- [4] Pastore, A.; Federici, G.; Bertini, E.; Piemonte, F.: Analysis of glutathione: Implication in redox and detoxification, *Clin. Chim. Acta*, 2003 **333**(1-2), 19-39 10.1016/S0009-8981(03)00200-6
- [5] Camera, E.; Picardo, M.: Analytical methods to investigate glutathione and related compounds in biological and pathological processes, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.*, 2002 **781**(1-2), 181-206 10.1016/S1570-0232(02)00618-9
- [6] Błońska-Sikora, E.; Oszczudłowski, J.; Witkiewicz, Z.; Wideł, D.: Glutathione: Methods of sample preparation for chromatography and capillary electrophoresis, *Chemik*, 2012 **66**(9), 936-942
- [7] Carru, C.; Zinellu, A.; Sotgia, S.; Marongiu, G.; Farina, M.G.; Usai, M.F.; Pes, G.M.; Tadolini, B.; Deiana, L.: Optimization of the principal parameters for the ultrarapid electrophoretic separation of reduced and oxidized glutathione by capillary electrophoresis, *J. Chromatogr. A*, 2003 **1017**(1-2), 233-238 10.1016/j.chroma.2003.08.020
- [8] Carru, C.; Zinellu, A.; Pes, G.M.; Marongiu, G.; Tadolini, B.; Deiana, L.: Ultrarapid capillary electrophoresis method for the determination of reduced and oxidized glutathione in red blood cells, *Electrophoresis*, 2002 **23**(11), 1716-1721 10.1002/1522-2683(200206)23:11<1716::AID-ELPS1716>3.0.CO;2-A
- [9] Serru, V.; Baudin, B.; Ziegler, F.; David, J.P.; Cals, M.J.; Vaubourdolle, M.; Mario, N.: Quantification of reduced and oxidized glutathione in whole blood samples by capillary electrophoresis, *Clin. Chem.*, 2001 **47**(7), 1321-1324
- [10] Maeso, N.; García-Martínez, D.; Rupérez, F.J.; Cifuentes, A.; Barbas, C.: Capillary electrophoresis of glutathione to monitor oxidative stress and response to antioxidant treatments in an animal model, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.*, 2005 **822**(1-2), 61-69 10.1016/j.jchromb.2005.05.015
- [11] Zhang, L.Y.; Sun, M.X.: Fast determination of glutathione by capillary electrophoresis with fluorescence detection using  $\beta$ -cyclodextrin as modifier, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.*, 2009 **877**(31), 4051-4054 10.1016/j.jchromb.2009.10.001
- [12] Musenga, A.; Mandrioli, R.; Bonifazi, P.; Kenndler, E.; Pompei, A.; Raggi, M.A.: Sensitive and selective determination of glutathione in probiotic bacteria by capillary electrophoresis-laser induced fluorescence, *Anal. Bioanal. Chem.*, 2007 **387**(3), 917-924 10.1007/s00216-006-0980-6
- [13] Zhang, J.; Hu, Z.; Chen, X.: Quantification of glutathione and glutathione disulfide in human plasma and tobacco leaves by capillary electrophoresis with laser-induced fluorescence detection, *Talanta*, 2005 **65**(4), 986-990 10.1016/j.talanta.2004.08.030

- [14] Jin, W.; Wang, Y.:Capillary zone electrophoresis with end-column amperometric detection for the determination of glutathione at a gold/mercury amalgam microelectrode without deoxygenation, *ANAL. CHIM. ACTA*, 1997 **343**(3), 231-239 10.1016/S0003-2670(96)00464-3
- [15] Jin, W.; Li, W.; Xu, Q.:Quantitative determination of glutathione in single human erythrocytes by capillary zone electrophoresis with electrochemical detection, *Electrophoresis*, 2000 **21**(4), 774-779 10.1002/(SICI)1522-2683(20000301)21:4<774::AID-ELPS774>3.0.CO;2-M
- [16] Lee, R.; Britz-McKibbin, P.:Differential rates of glutathione oxidation for assessment of cellular redox status and antioxidant capacity by capillary electrophoresis-mass spectrometry: An elusive biomarker of oxidative stress, *Anal. Chem.*, 2009 **81**(16), 7047-7056 10.1021/ac901174g
- [17] Bayle, C.; Caussé, E.; Couderc, F.:Determination of aminothiols in body fluids, cells, and tissues by capillary electrophoresis, *Electrophoresis*, 2004 **25**(10-11), 1457-1472 10.1002/elps.200305874
- [18] Winther, J.R.; Thorpe, C.:Quantification of thiols and disulfides, *Biochim. Biophys. Acta*, 2014 **1840**(2), 838-846 <https://doi.org/10.1016/j.bbagen.2013.03.031>

### **P3 GLYCOSIMILARITY INDEX FOR BIOTHERAPEUTICS**

ANDRAS GUTTMAN<sup>1,2,3</sup>, BEATA BORZA<sup>2,3</sup>, MARTON SZIGETI<sup>2,3</sup>, AKOS SZEKRENYES<sup>2</sup>, LASZLO HAJBA<sup>3</sup>

1 Sciex, Brea, CA, USA

2 Horváth Csaba Laboratory of Bioseparation Sciences, University of Debrecen, Hungary;

3 MTA-PE Translational Glycomics Research Group, Research Institute for Biomolecular and Chemical Engineering, University of Pannonia, Veszprem, Hungary

#### **Abstract**

Recent expiration of numerous protein therapeutics opened up the opportunity for the development of biosimilars. Critical quality attributes (e.g., post-translational modifications of recombinant biotherapeutics) have great impact on clinical efficacy and safety of biosimilars. Therefore, regulatory authorities require comprehensive analysis of protein structure, post-translational modifications and biological activity during the development process of a biosimilar product. Since most recombinant protein biologics and consequently their biosimilar counterparts are glycosylated, information about their carbohydrate moieties represent an important information to demonstrate similarity. Glycosylation could have a significant impact on the biological activity, effector functions, and immunogenicity of the drug products. The first part of this presentation summarizes the attributes of biosimilarity, considering the regulatory guidelines and the relevant statistical aspects (e.g., biosimilarity index). The second part discusses glycosylation as one of the important attributes of biosimilarity in detail. Based on the traditional average bioequivalence criterion, the glycosimilarity index is defined as a subset of the biosimilarity index. The test product is similar to the reference product from this important post translational modification point of view if the glycosimilarity index falls in the range of 90% - 111%. Practical examples of glycosimilarity index calculation of several originator and their biosimilars will be shown based on capillary electrophoresis analysis of their glycosylation patterns.

#### **Acknowledgment**

The authors gratefully acknowledge the support of the Momentum (Lendület) grant # 97101 of the Hungarian Academy of Sciences (MTA-PE Translational Glycomics) and the National Research, Development and Innovation Office (NKFIH) (K 116263) grants of the Hungarian Government. This work was also supported by the BIONANO\_GINOP-2.3.2-15-2016-00017 project.

## P4 SEMIAUTOMATION OF LIQUID HANDLING FOR MICROELECTROMEMBRANE EXTRACTION OF COMPLEX SAMPLES

MILOŠ DVOŘÁK\*, ANDREA ŠLAMPOVÁ, AND PAVEL KUBÁŇ

Institute of Analytical Chemistry of the Czech Academy of Sciences, v. v. i., Veveří 97, CZ-60200 Brno, Czech Republic

**Abstract** – Application of a programmable pump directly connected with an extraction unit was examined for micro-electromembrane extractions ( $\mu$ -EMEs) of complex samples across free liquid membrane (FLM). Performance of liquid handling and repeatability of withdrawn volumes were investigated and excellent results (relative standard deviation (RSD) values lower than 3%) were obtained for handling  $\mu$ L volumes of  $\mu$ -EME operational solutions. A simple program sequence for semiautomated handling of three adjacent phases is shown and discussed, which can be easily modified for use with standard or complex samples. The semiautomated system was practically applied for  $\mu$ -EME of four basic drugs from biological fluids. The  $\mu$ -EME step was followed by analysis of acceptor solution by capillary electrophoresis demonstrating extraction recoveries above 70% and repeatability of the analytical method below 10%.

**Keywords:** free liquid membrane, automation, micro-electromembrane extraction

### 1. Introduction

Micro-electromembrane extraction ( $\mu$ -EME) was introduced as a novel miniaturized technique for extractions of complex samples in 2014 [1]. A free liquid membrane (FLM), usually a water immiscible phase, is sandwiched between two aqueous solutions to form a three phase  $\mu$ -EME system. All solutions are free liquids and are stabilized by capillary forces in a low-bore capillary tubing.

Advantages of  $\mu$ -EME across FLM are reduced consumption of samples and organic solvents, higher variability of membrane thickness/shape, higher stability of the membrane and suitability for real-time monitoring of extraction process [2]. The low consumption of sample can be favourable in extractions of biological fluids, which are usually available in limited amounts [3]. On the other hand, labour intensive procedures and manual handling of microliter volumes of operational solutions are its major disadvantages, which may have direct bearing on the extraction method performance.

Liquid handling with semiautomated systems was reported for microchip EME [4] and dynamic EME [5]. Mechanical pumps continuously delivered sample and acceptor solution to each side of a supported liquid membrane (SLM), which was in form of a porous solid material impregnated with an organic solvent. Manual replacement of SLM was, however, required between EMEs thus disabling full automation of the extraction process.

This contribution describes a system for semiautomated handling of all three immiscible phases in  $\mu$ -EME of basic drugs from human body fluids. A special attention is focused onto liquid handling of protein-containing body fluids. A simple program is presented, which eliminates transfer of proteins to acceptor solutions, is suitable for efficient extraction of model basic drugs from human body fluids and the presented work opens new possibilities for automation of  $\mu$ -EME techniques.

### 2. Experimental

#### 2.1. Chemicals and reagents

All chemicals were purchased from Lach-Ner (Neratovice, Czech Republic), Sigma (Steinheim, Germany) and Fluka (Buchs, Switzerland) and were of reagent grade. Deionized water (DI) prepared by exchange of ions in a mixed-bed ion exchanger water purification system G 7749 (Miele, Gütersloh, Germany) with resistivity higher than 18 M $\Omega$ ·cm was used. Stock solution of 1.5 M NaCl was prepared from pure NaCl (Lach-Ner) in DI water. Stock solutions of basic drugs nortriptyline hydrochloride, papaverine hydrochloride, haloperidol and loperamide hydrochloride (1 gL<sup>-1</sup>, Sigma) were prepared in methanol (Lach-Ner). Standard solutions of the four basic drugs were prepared from these stock solutions by mixing with hydrochloric acid (Lach-Ner). Background electrolyte (BGE) solutions for capillary electrophoresis (CE) analyses were prepared in DI water and consisted of 15 mM sodium dihydrogen

\*\*\*Dr. Miloš Dvořák, e-mail: dvorak@iach.cz, Tel: +420 532290121, Fax: +420 541212113, Institute of Analytical

Chemistry of the Czech Academy of Sciences, v. v. i., Veveří 97, CZ-60200 Brno, Czech Republic

phosphate and 15 mM ortho-phosphoric acid (Lach-Ner); pH of BGE solutions was 2.23. 1-ethyl-2-nitrobenzene (ENB, Fluka) was used as FLM in  $\mu$ -EME experiments.

## 2.2. Samples, instruments and measurements

Plasma samples were purchased as lyophilised powders from Sigma and were diluted with DI water according to a procedure recommended by the manufacturer. The plasma samples were stored at  $-20^{\circ}\text{C}$  and were allowed to warm up to the ambient temperature before they were diluted with hydrochloric acid and used for  $\mu$ -EME.

A high voltage power supply unit (Spellman CZE1000R Start Spellman, Pulborough, UK) was connected with UV-Vis detector (Spectra 100, Spectra Physics, U.S.A.) in a home-made CE system. Electric potential of + 18 kV was applied at the injection side of the separation capillary for all runs and detection wavelength was 200 nm. Data were collected using Orca-2800 (Ecom, Prague, Czech Republic) data acquisition system connected to a personal computer with Ecomac (Ecom) software. CE-UV analyses of basic drugs were performed in 75/375  $\mu\text{m}$  ID/OD fused silica (FS) capillary with total length of 55 cm and effective length of 10 cm. FS capillaries were purchased from Polymicro Technologies, Phoenix, AZ, USA. Initial preconditioning procedure employed flushing with 1 M NaOH, DI water and BGE solution for 5 min each. Between two analyses, the capillary was flushed with BGE solution for 1 min.

Injections of  $\mu\text{L}$  sample volumes were carried out from polypropylene (PP) micropipette tips (0.1 – 10  $\mu\text{L}$ , Eppendorf, Hamburg, Germany). The PP tip with the solution was attached (in vertical position) to a movable arm, which elevated the tip to the injection position and ensured contact between the capillary and the injected solution. The capillary outlet end was positioned 10 cm below the capillary injection end and the sample was injected hydrodynamically for 15 s into the capillary. After sample injection, capillary outlet and inlet were placed into vials with BGE solution and high voltage was applied. All CE experiments were performed at ambient temperature of  $25^{\circ}\text{C}$ .

A schema of the instrumental set-up for  $\mu$ -EME system with semi-automated liquid handling is depicted in *Fig.1*. A programmable syringe pump

(NE-1000, New Era Pump Systems Inc., Farmingdale, NY, USA) was equipped with a 25  $\mu\text{L}$  glass syringe (1705RN, Hamilton, Bonaduz, Switzerland). The pump was operated in withdrawal and infusion mode for filling of extraction units with respective solutions and for collection of operational solution after  $\mu$ -EMEs, respectively. Piston diameter was adjusted to 0.73 mm. For details on programming the sequence see Table 1. PP micropipette tips (0.5 – 10  $\mu\text{L}$ , Kartell, Noviglio, Italy) were used as the extraction units. A flat bottom needle of the Hamilton syringe and the PP extraction unit were connected using a 1 cm long piece of flexible Tygon tubing (0.25 mm ID/1.6 mm OD, Gilson, Villers le Bel, France) and 1 cm long piece of flexible silicone tubing (1/3 mm ID/OD, Gumex, Strážnice, Czech Republic). A tubular copper wire (0.15 mm thick, 5 cm long) was thread between the Tygon and silicone tubing, protruded by 3 cm into the extraction unit and formed one electrode of the  $\mu$ -EME system. On connecting the pump with the  $\mu$ -EME unit, the Cu electrode was directed to the wider part of the unit and the outlet of the silicone tubing was firmly pressed against the unit to ensure a leak-free connection.

$\mu$ -EME operational solutions were filled into extraction units using a program for withdrawal of specific volumes of donor solution, FLM and acceptor solution interspaced by wait periods of 15 s. Program for withdrawal of plasma sample is shown in Table 1.

A second electrode (0.25 mm thick tubular Pt wire, 99.95%, Advent, Oxford, England) was inserted into the narrow end of the extraction unit after the three phases were formed. To avoid evaporation of the phases, the tip was closed with a protective sleeve [6]. The electrodes were connected to a d.c. power supply EV 231 (Consort, Turnhout, Belgium); anode was the Cu electrode in donor solution, cathode was the Pt electrode in acceptor solution and extraction voltage was switched on. The extraction unit was positioned horizontally during  $\mu$ -EME with no agitation. All  $\mu$ -EME experiments were performed at ambient temperature of  $25^{\circ}\text{C}$ . After  $\mu$ -EME, the syringe pump was switched to the infusion mode and acceptor solution was collected into the narrower end of a clean micropipette tip (Eppendorf) and was used for CE analysis. Organic phase was collected into another tip and was discarded. Donor solution

was collected into a third tip and was used for CE analysis. Infusion flow rate was 15  $\mu\text{L}/\text{min}$ .

Performance of the syringe pump was examined with high precision scales Kern 770-60 (Kern & Sohn GmbH, Balingen, Germany) with 0.01 mg readout.

Extraction recoveries (ER) were calculated according to equation reported in reference [3].

### 3. Results and Discussion

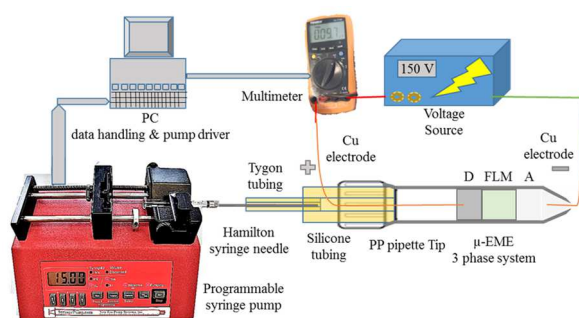


Figure 1. Semiautomated set-up for  $\mu\text{-EME}$ .

#### 3.1. Semiautomated liquid handling

Several materials were examined for connection of the pump system with the micro-extraction unit and for accommodation of the  $\mu\text{-EME}$  electrode. These included use of non-flexible (for example PP tips and PTFE tubing) and flexible (for example Tygon and silicone tubing) consumables. Using of the non-flexible materials is not recommended since leaks and non reproducible withdrawal of liquids was observed. This was attributed to microspaces at the connection point between the pump and the microextraction unit and in the puncture for  $\mu\text{-EME}$  electrode. The final arrangement included flexible Tygon/silicone tubing, which ensured leak-proof connections and easy manipulation and is depicted in Fig.1. Details on materials and connections can be found in Experimental part.

Precision of pump system was examined by withdrawal of various volumes of DI water. 1.3, 2.5 and 5  $\mu\text{L}$  were selected as typical  $\mu\text{-EME}$  volumes and repeatability of the withdrawal process is summarized in Fig.2. Empty unit was weighed, defined volume of DI water was withdrawn and the unit was weighed again. Volume of withdrawn DI water was calculated by subtracting weight of empty unit from the weight of filled unit divided by DI water density at 25°C. Relative standard

deviation (RSD) values for 6 subsequent withdrawals were less than 3%.

#### 3.2. Semiautomated handling of body fluids

The pump program can be entered directly from the pump keyboard or using a personal computer. In

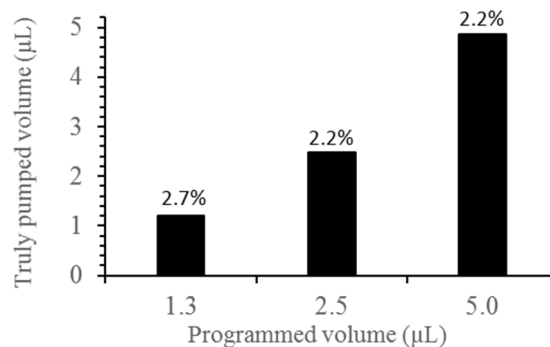


Figure 2. Programmed and truly pumped volume for  $\mu\text{-EME}$  system. For conditions see experiments.

our experiments, the program was entered directly from the pump keyboard due to the possibility for fast and simple program modifications. The 15 s of pump inactivity after withdrawal steps, included in the program, were used for manual changing of reservoirs with operational solutions. All operational solutions were withdrawn and infused at a flow rate of 15  $\mu\text{L}/\text{min}$ . This flow rate provided sufficient precision of pumping and the filling procedure took less 2 minutes depending on volumes of solutions. Withdrawal of biological fluids were examined with human plasma samples. Plasma was withdrawn into extraction unit (1.3  $\mu\text{L}$ ) followed by withdrawal of FLM (2.5  $\mu\text{L}$ ) and of acceptor solution (1.3  $\mu\text{L}$ ). The acceptor solution was collected and analysed by CE. Adsorption of plasma proteins to the PP unit and subsequent transfer to acceptor solution was evidenced by presence of protein peaks in CE electropherograms and by deteriorated CE performance. To avoid transfer of proteins to the acceptor solution, the unit tip was flushed with  $2 \times 2.0 \mu\text{L}$  of DI water (Phases 5-10) before the final withdrawal of acceptor solution. Presence of proteins in CE runs was not observed after the additional treatment. The final



program suitable for semiautomated filling of highly complex samples is summarized in *Table 1*.

For standard solutions and samples with lower complexity (urine, saliva, etc.) Phases 5-10 can be deleted from the program.

*Table 1.* Pump program for filling the microextraction unit with three phases for  $\mu$ -EME of human plasma.

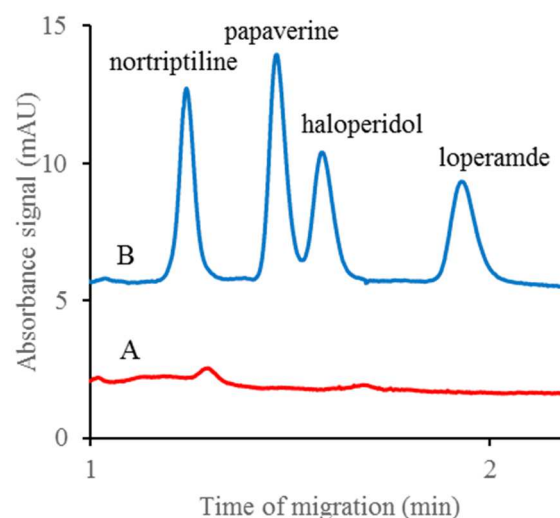
Phase	Function	Rate $\mu\text{L}/\text{min}$	Volume $\mu\text{L}$	Direction
1	RATE	15	1.3	Withdraw
2	PS:15			
3	RATE	15	2.5	Withdraw
4	PS:15			
5	RATE	15	2.0	Withdraw
6	RATE	15	2.0	Infuse
7	PS:15			
8	RATE	15	2.0	Withdraw
9	RATE	15	2.0	Infuse
10	PS:15			
11	RATE	15	1.3	Withdraw
12	STOP			

### 3.3. $\mu$ -EME of basic drugs from body fluids

Volumes of operational solutions (1.3  $\mu\text{L}$  of donor solution, 2.5  $\mu\text{L}$  of FLM and 1.3  $\mu\text{L}$  of acceptor solution) were selected as the minimum volumes providing stable extraction conditions at extraction potential of 150 V and time of 10 min. They were semiautomatically withdrawn into the extraction unit using the program reported in *Table 1*. Donor solutions and samples were acidified by HCl to obtain final concentration of 10 mM HCl. Final concentration of HCl in acceptor solution was 25 mM. Both solutions ensured complete ionization of basic drugs and thus their efficient  $\mu$ -EME and pH of the solutions was not affected by electrolysis [7]. HCl at the above concentrations had not impact on peak shapes and sharp and well distinguished peaks were observed for all CE runs.

Blank plasma (plasma diluted 1:1 with 20 mM HCl) was extracted first and no peaks related to the four basic drugs were detected in the electropherogram (see *Fig.3* trace A). The drug-free plasma samples were subsequently spiked with the four basic drugs (10  $\text{mgL}^{-1}$ ) and extracted at the same  $\mu$ -EME conditions. CE analysis of acceptor solution after  $\mu$ -EME of spiked human plasma is depicted in *Fig.3* trace B and confirms efficient extraction of the drugs from human plasma. Comparable extraction efficiency was obtained for  $\mu$ -EME of the drugs from standard solutions and

from other human body fluids spiked with the drugs (urine, saliva, serum).



*Figure 3.* Electropherograms showing human plasma after  $\mu$ -EME. A: plasma diluted 1:1 with 20 mM HCl (blank). B: plasma diluted 1:1 with 20 mM HCl containing 20  $\text{mgL}^{-1}$  of basic drugs (spiked plasma). CE and  $\mu$ -EME conditions see Experimental part and Chapter 3.3, respectively.

The semiautomated  $\mu$ -EME system was characterized by excellent analytical performance. Extraction recoveries for model analytes were higher than 70%, repeatability of  $\mu$ -EME expressed as RSD values of peak areas in CE measurements was better than 10% and linear relationship (coefficients of determination better than 0.99) was obtained in concentration range of 0.5 – 10  $\text{mgL}^{-1}$ .

## 4. Conclusion

Simple system for semiautomation of liquid handling in  $\mu$ -EME systems was presented. A programmable syringe pump was directly connected with  $\mu$ -EME microextraction unit for automated withdrawal and infusion of  $\mu$ -EME operational solutions. Pump program was easily tailored for handling standard solution or protein-containing complex samples prior to their  $\mu$ -EME. Leak-free connections between pump syringe and  $\mu$ -EME system granted satisfactory handling of  $\mu\text{L}$  volumes of  $\mu$ -EME operational solutions, and guaranteed good reproducibility and extraction recovery of  $\mu$ -EME across FLM.

## Acknowledgement

Financial support from the Czech Academy of Sciences (Institute Research Funding RVO:68081715) and the Grant Agency of the Czech Republic (Grant No. 16-09135S) is gratefully acknowledged.

## REFERENCES

- [1] Kubáň, P.; Boček, P.: Micro-electromembrane extraction across free liquid membranes. Extractions of basic drugs from undiluted biological samples. *J. Chromatogr. A.*, 2014 **1337**, 32-39, doi:10.1016/j.chroma.2014.02.046
- [2] Huang, C.; Chen, Z.; Gjelstad A.; Pedersen-Bjergaard S.; Shen X.; Electromembrane extraction, *TrAC - Trends Anal. Chem.*, 2017 **95**, 47-56, doi:10.1016/j.trac.2017.07.027
- [3] Pedersen-Bjergaard, S.; Rasmussen, K.E.: Electrokinetic migration across artificial liquid membranes. New concept for rapid sample preparation of biological fluids. *J. Chromatogr. A*, 2006 **1109**, 183-190, doi:10.1016/j.chroma.2006.01.025
- [4] Petersen, N.J.; Foss, S.T.; Jensen, H., Hansen, S.H.; Skonberg, C.; Snakenborg, D.; Kutter, J.P.; Pedersen-Bjergaard, S.: On-Chip Electro Membrane Extraction with Online Ultraviolet and Mass Spectrometric Detection. *Anal. Chem.* 2011 **83**, 44-51, doi: 10.1021/ac1027148
- [5] Drouin, N.; Rudaz, S.; Schappler, J.: Dynamic-Electromembrane Extraction: A Technical Development for the Extraction of Neuropeptides, *Anal. Chem.* 2016 **88**, 5308-5315. doi: 10.1021/acs.analchem.6b00559
- [6] Kubáň, P.; Seip, K.F.; Gjelstad, A.; Pedersen-Bjerggaard, S.: Micro-electromembrane extraction using multiple free liquid membranes and acceptor solutions – Towards selective extractions of analytes based on their acid-base strength. *Anal. Chim. Acta*, 2016 **943**, 64-73, doi:10.1016/j.aca.2016.09.022
- [7] Kubáň, P.; Boček, P.: The effects of electrolysis on operational solutions in electromembrane extraction: The role of acceptor solution. *J. Chromatogr. A*, 2015 **1398**, 11-15, doi: 10.1016/j.chroma.2015.04.024

# P5 DEVELOPMENT OF GRADIENT RP-HPLC METHOD FOR CHARACTERIZATION OF AMINO ACID COMPOSITION OF DEGRADATION PRODUCTS OF HUMIC ACIDS ISOLATED FROM PEAT AND SOIL AFTER PRE-COLUMN DERIVATIZATION

RÓBERT GÓRA,<sup>1\*</sup> MILAN HUTTA,<sup>1</sup> ERIK BEŇO,<sup>1</sup> AND NATÁLIA BIELČÍKOVÁ<sup>1</sup>

<sup>1</sup> Department of Analytical Chemistry, Comenius University in Bratislava, Ilkovičova, u. 6, Bratislava, 84215, Slovakia

Abstract – The aim of the present work was the development of RP-HPLC method using a monolithic silica column for determination of amino acids derivatized with ethoxymethylenemalonate (DEEMM). The created products are highly stable and offer characteristic spectra in UV region with maximum at 280 nm. The developed method was applied to determination of selected amino acids in the samples of degradation products of naturally occurred macromolecules after hydrolysis with concentrated hydrochloride acid solution at 110 °C.

**Keywords:** Amino acids, Humic substances, Diethylethoxymethylenemalonate, RP-HPLC

## 1. Introduction

Soil humic acids (HA) arise mainly due to (bio)chemical degradation of plant and animal residues and also from microbial synthetic activity. HS constitute a significant fraction of the soil organic matter (SOM) (about 20% of the total SOM) [1]. Approximately 20-50% of total nitrogen (N) occurring in soil organic matter (SOM) was found in amino acids forms, which are one of the most important natural compounds. Amino acids (AA) participate in the formation of peptide bonds, which can be release by acid hydrolysis [2]. Majority of amino acids occurs in soils in bound form, e.g., in huminopeptide structures. They are usually bound to the central core (nucleus) of HA or FA (fulvic acid), thus protected from fast degradation by microorganisms [3].

Liquid chromatography belongs to the most employed analytical methods for determination of amino acids. Its drawback, however, is the limits of options for direct detection, as most amino acids do not exhibit fluorescence and only a few contain structural moieties with chromophores. For this reason most of the chromatographic methods designed for this purpose implements pre-column or post-column derivatization. On the basis of available information, diethyl ethoxymethylmalonate (DEEMM) appears to be the reagent of choice, converting amino acids and compounds with a primary or secondary amino group according to reaction scheme in Figure 1. The created products are highly stable and offer characteristic spectra in ultraviolet region with maximum at 280 nm [4].

In the present work we focused on the development of RP-HPLC method for the determination of selected AA, potentially present in samples of degradation products of naturally occurred macromolecules (humic and fulvic acid) after acidic hydrolysis using pre-column derivatization with less frequently used reagent diethylethoxymethylene-malonate (DEEMM).

## 2. Experimental

All chromatographic experiments were carried out by the HPLC system LaChrom (Merck-Hitachi, Darmstadt, Germany) equipped with the quaternary pump L-7100, autosampler L-7200, column oven L-7300 (with an accuracy of  $\pm 0.5^\circ\text{C}$ ), diode-array detector L-7450, fluorescence detector (FLD) L-7480, solvent degasser L-7612, interface D-7000, and software HSM v. 4.1 was used.

The chromatographic separation of AA samples was performed using an analytical column Chromolith Performance RP-18e (100×4.6 mm) with the guard column (Chromolith RP-18e (5×4.6 mm) Guard Cartridge). The flow rate was

maintained at 1.00 ml min<sup>-1</sup>. Mobile phase consisted of a mixture of phosphate buffer solution (pH 3.6) or trifluoroacetic acid solution (20 mmol.L<sup>-1</sup>, pH 5.0) and methanol. Column oven temperature was maintained at 35.0 °C ± 0.1°C. Injection volume 10 µl was injected by the autosampler. Wavelength range of DAD was set to 280 - 800 nm and monitored wavelength was set to 280 nm.

HPLC-grade methanol 99% for LC, trifluoroacetic acid 99% p.a. and sodium dihydrogen phosphate p.a. for mobile phase preparation and hydrochloride acid 33% (HCl) required for acidic hydrolysis of HA samples were obtained from Merck (Darmstadt, Germany). Amino acid standards (l-glutamine, l-cysteine, l-proline, l-tryptophan, l-tyrosine, l-histidine, l-leucine, l-isoleucine, l-phenylalanine, l-alanine, l-aspartic acid, l-arginine, l-asparagine, glycine, l-serine, l-valine, l-methionine, l-threonine, l-lysine, and l-glutamic acid) were purchased from Merck (Darmstadt, Germany). Derivatization agent diethyl ethoxymethylenemalonate and sodiumtetraborate were purchased from Sigma Aldrich (St. Louis, USA). Water for gradient HPLC was prepared by Labconco Pro-PS unit (Labconco, Kansas City, USA) and purified by Millipore Simplicity system (Molsheim, France).

Target group of humic acids was isolated from soil and peat accordance with the procedures published by Kandráč et al. [5].

### 3. Results and Discussion

#### 3.1. Experiments

Samples of HA were decomposed by hydrolysis of 6 mol.L<sup>-1</sup> hydrochloric acid (HCl) at 110°C and degradation products of HA samples were derivatized with DEMM, preferred mainly due to its excellent properties. Among those certainly is formation of thermodynamically and kinetically stable products with a characteristic spectrum band at 280 nm, which is an important prerequisite for the design of robust RP-HPLC method using spectrophotometric detection.

In Figure 1 is illustrated a typical chromatographic profile of derivatization agent DEEMM at 254 and 280 nm. As from chromatographic profiles result,

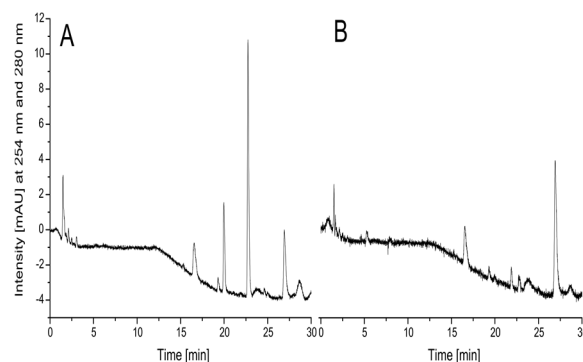


Figure 1. Chromatographic profiles of DEEMM recorded at 254 (A) and 280 nm (B) obtained using gradient elution with mixing of 20 mmol l<sup>-1</sup> TFA at pH 5.0 (A) and methanol (B), injection volume 10µl, column temperature 35 ° C, flow rate of 1 ml.min<sup>-1</sup>

DEEMM has a relatively high response at a wavelength of 254 nm and offers total of 5 system peaks, on the other hand the response DEEMM at a wavelength of 280 nm, we was used in the determination of AA, shows only one peak with relatively low response beside to responses of DEEMM derivatized AA, for which the wavelength 280 nm are specific and characteristic.

In the introductory part of the work, we focus on optimizing conditions for the separation of amino acids, depending on the composition of the mobile phase. Based on our preliminary measurements, trifluoroacetic acid (TFA) was chosen as an aqueous part of mobile phase, due to its advantageous properties and the potential possibility of using MS detection in the future. At the beginning, we have focused on the influence of pH of the mobile phase (TFA ranging pH from 3.00 to 7.00) to separation process. Obtained results showed that the optimum pH buffer TFA in the mobile phase for the

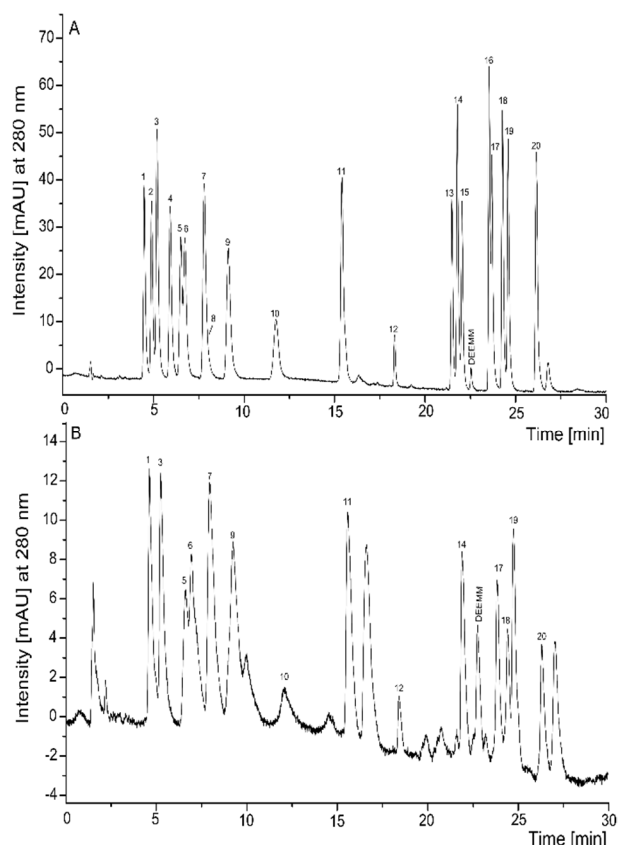


Figure 2. Chromatograms of (a) AA standard solution and (b) AA content in the samples of hydrolysates of HA isolated from peat (locality Cérová) obtained at 280 nm. Peak identification: 1–aspartic acid, 2–asparagine, 3–serine, 4–glutamine, 5–glutamic acid, 6–histidine, 7–glycine, 8–proline, 9–threonine, 10–arginine, 11–alanine, 12–tyrosine, 13–methionine, 14–valine, 15–tryptophan, 16–cysteine, 17–phenylalanine, 18–isoleucine, 19–leucine, 20–lysine.

separation of a standard AA and AA present in the samples of HA hydrolysates at pH 5.00. In the following step, we focused to study the impact of the ionic strength of aqueous constituent of mobile phase to separation of AA using monolithic type column Chromolith Performance RP-18e (100x4.6 mm) and for the other measurements we choose TFA concentration of 20 mmol.L<sup>-1</sup>.

#### 4. Conclusion

Described method was successfully applied to determination of the amino acid composition in degradation products of selected of humic acid samples from different origin. Both analysed samples of humic acids indicated higher abundance of amino acids. Glycine (in samples isolated from peat), Aspartic acid (in samples isolated from soil), Threonine and Alanine were the major AA in samples of degradation products of selected of humic acid.

#### Acknowledgement

The research was supported by project VEGA 1/0899/16 and Grant UK/110/2017..

#### REFERENCES

- [1] Allard B., *Geoderma* 130 (2006) 77.
- [2] Ariza J.L.G., Portero M.J.V., Daza V.B., *Anal. Chim. Acta* 540 (2005) 221.
- [3] Szajdak L., Jezierski A., Cabrera M.L., *Org. Geochem.* 34 (2003) 693.
- [4] Riin R., Koit H., *Anal. Chim. Acta* 672 (2010) 79.
- [5] Kandráč J., Hutta M., Foltin M., *J. Radioanal. Nucl. Chem., Articles*, 208 (1996) 587.

# P6 STUDY OF VARIOUS PARAMETERS INFLUENCING CONTENT OF EXHALED BREATH CONDENSATE WITH IMPORTANCE FOR DIAGNOSIS OF GASTROESOPHAGEAL REFLUX DISEASE

MICHAL GREGUŠ,<sup>1,2\*</sup> PAVOL ĎURČ,<sup>1,2</sup> JÚLIA LAČNÁ,<sup>1,2</sup> FRANTIŠEK FORET<sup>1,3</sup> AND PETR KUBÁŇ<sup>1,2,3</sup>

1 Department of Bioanalytical Instrumentation, CEITEC Masaryk University, Veveří 97, Brno, 602 00, Czech Republic

2 Department of Chemistry, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic

3 Department of Bioanalytical Instrumentation, Institute of Analytical Chemistry of the Czech Academy of Sciences, v.v.i., Veveří 97, 602 00 Brno, Czech Republic

**Abstract** – In this work, various parameters that influence the ionic content and pH of exhaled breath condensate with importance to non-invasive diagnosis of gastroesophageal reflux disease were studied. Exhaled breath condensate samples were collected using a miniature and inexpensive sampling device. Capillary electrophoresis with contactless conductometric detection was used for monitoring the ionic content of exhaled breath condensate. Background electrolyte composed of 20 mM 2-(N-morpholino) ethanesulfonic acid, 20 mM L-histidine, 2 mM 18-crown-6 and 30  $\mu$ M cetyltrimethylammonium bromide allowed fast separation of anions and cations, both in less than 2 minutes. Possibility of exhaled breath condensate contamination by saliva is discussed in detail. Day-to-day repeatability (n=5) of ionic content and pH of exhaled breath condensate was studied and was satisfactory, reflecting mainly the physiological variability.

**Keywords:** capillary electrophoresis, exhaled breath condensate, ionic analysis, pH, gastroesophageal reflux disease

## 1. Introduction

Gastroesophageal reflux disease (GERD) is a disease caused by backflow of gastric contents into the esophagus due to the failure of physiological antireflux mechanisms and can lead to symptoms such as chronic cough, globus sensation, laryngitis, pharyngitis, rhinosinusitis, otitis media, bronchial asthma, chronic obstructive pulmonary disease, sleep apnea and noncardiac chest pain [1,2]. Currently, there is no suitable, non-invasive diagnostic method applicable for GERD in clinical practice. Nowadays, the golden standard in diagnosis of GERD is a 24-hour pH-multichannel intraluminal impedance measurement (pH-MII) that is rather invasive [3]. Non-invasive sampling is currently increasing in importance. Exhaled breath condensate (EBC) as one of non-invasive samples can be easily obtained by cooling and subsequent condensation of exhaled breath. The EBC is composed mainly of water, however, it also contains volatile and non-volatile compounds (inorganic ions, organic acids) that can be useful as biomarkers of various diseases [4].

Determination of ions in EBC samples can be easily performed by capillary electrophoresis (CE). The main advantage of this method is that it is able to cope with a minute volume of samples and analyses are often very rapid. Another parameter that is often measured the diagnosis of GERD is pH,

measured by a conventional pH-MII probe, which is both invasive and costly. pH can be also measured in collected samples of EBC [5] that is non-invasive and cheaper. Monitoring of various parameters of exhaled breath condensate (EBC) in patients suffering from symptoms of extraesophageal/gastroesophageal reflux (EER/GER) is an attractive, simple and non-invasive approach that could be used as a surrogate to other, more invasive diagnostic methods. However, to obtain relevant results, various parameters that can affect the results, such as sampling procedure, effect of food and drinks and intraday variability of individuals have to be studied prior to the use of EBC sampling in diagnostics.

## 2. Experimental

### 2.1. Instrumentation

A purpose-built CE instrument was used for all CE analyses. The separation voltage was provided by a high-voltage power supply unit (DX250, EMCO high voltages, Sutter Creek, CA, USA). A custom made contactless conductivity detector (C4D, Ver. 5.06, ADMET s.r.o., Prague, Czech Republic) operating at a frequency of 1.8432 MHz and voltage 50  $V_{p-p}$  and 24-bit A/D converter (ORCA 2800, ECOM s.r.o., Prague, Czech Republic) were used for detection of separated analytes and data

\*\*Correspondence: Michal Greguš, gregus@mail.muni.cz

collection, respectively. CE analyses were performed in fused-silica separation capillaries (50  $\mu\text{m}$  inner diameter (ID), 375  $\mu\text{m}$  outer diameter (OD), 40 cm total length, 17 cm effective length, Microquartz GmbH, Munich, Germany). Injection of standard solution and samples was performed hydrodynamically. Two Pt wires (0.5 mm OD, 3 cm length, Advent Research Materials Ltd., Eynsham, England) were inserted in the electrolyte vials to serve as electrodes. All CE experiments were performed at ambient temperature.

For pH measurement of the EBC samples,  $\text{CO}_2$  present in these samples was removed by a stream of  $\text{N}_2$ -gas (nitrogen generator, RP-ZE-N2L3000). pH was measured using a pH-microelectrode (MI-410 Micro-Combination pH Probe, Microelectrodes, Inc., Bedford, New Hampshire, USA) and pH-meter (Orion Star™ A111 pH Benchtop Meter, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

EBC samples were collected from the volunteers by EBC sampler previously developed in our group [6,7]. Briefly, the sampler for collection of EBC was constructed from a 2-mL syringe (B. Braun Melsungen AG, Melsungen, Germany) and a 5-cm long hollow aluminum cylinder (see Fig.1) of 2.5 cm outer diameter (OD) with inner diameter (ID) precisely matching the outer diameter of the syringe was used for efficient cooling of the walls. The cylinder was kept in a deep freezer at  $-17^\circ\text{C}$  and an insulation sleeve was used during collection to maintain its constant temperature for the whole duration of the sampling procedure. Another aim of this insulation sleeve was to protect the person during the sample collection from frostbite. Straw (purchased from a local store) with OD 6 mm and wall thickness 0.2 mm was used to exhale the air through the sampler. The end of the syringe was enclosed with a Parafilm septum to avoid EBC loss.



Figure 1. The photo of the consumables used to build a miniature EBC sampling device.

## 2.2. Chemicals

All chemicals were of reagent grade, and deionized (DI) water was used for stock solution preparation and dilutions. Stock solutions (10 mmol/L) of inorganic anions and cations were prepared from their sodium and chloride salts ( $\text{NaCl}$ ,  $\text{NaNO}_3$ ,  $\text{NaNO}_2$ ,  $\text{Na}_2\text{SO}_4$ ,  $\text{KCl}$ ,  $\text{CaCl}_2$ ,  $\text{MgCl}_2$ ), except for ammonium, the stock solution of which was prepared from ammonium fluoride (all from Pliva-Lachema, Brno, Czech Republic). The stock solutions of organic acid anions were prepared from lithium acetate dihydrate, lithium lactate, propionic acid and butyric acid (all from Sigma-Aldrich, Steinheim, Germany).

The background electrolyte (BGE) was prepared daily by dilution of 100 mmol/L stock solutions of 2-(N-morpholino)ethanesulfonic acid (MES), L-histidine (HIS), 18-crown-6 and 10 mmol/L stock solution of hexadecyltrimethylammonium bromide (CTAB) (all from Sigma-Aldrich, Steinheim, Germany).

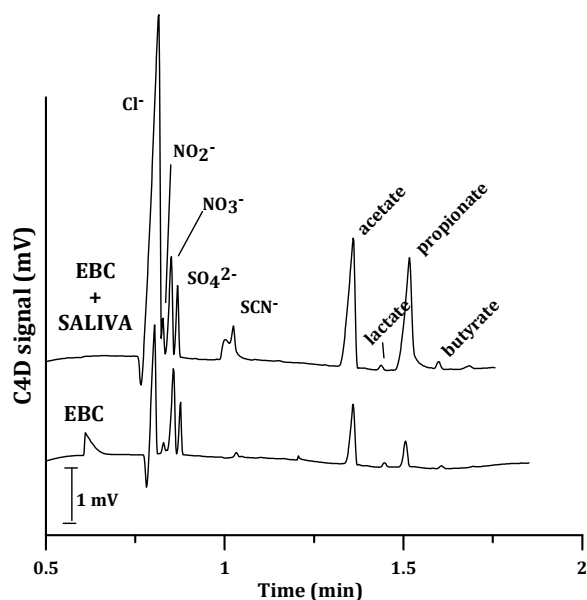
## 2.3. Capillary conditioning

Prior to the first use, the separation capillary was preconditioned by flushing with 0.1 M NaOH for 30 min, DI water for 30 min and BGE solution for 20 min. Between two successive injections, the capillary was flushed with BGE solution for 1 min. At the end of a working day, the capillaries were washed with DI water for 15 min and stored in DI water overnight.

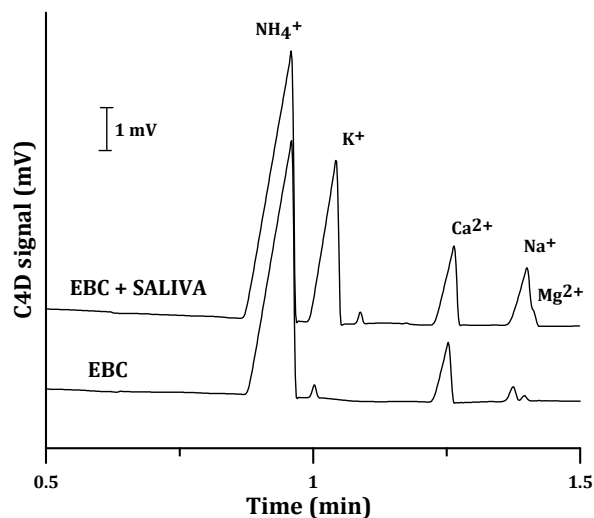
### 3. Results and Analysis

#### 3.1. EBC sampling procedure

The collection of EBC consists of several steps: deep exhalation until the lungs were as empty as possible, following quick inhalation (1-2 s) and finally slow complete exhalation into the sampling device (8-10 s). This procedure was suggested by Almstrand *et al.* [8] and yielded the higher concentration of respiratory droplets and therefore the highest concentrations of non-volatile substances present in EBC. The volunteer was asked to exhale three times into the sampling device, based on the proposed procedure. After the EBC sampling had been completed, the condensate from the syringe walls was swept with the syringe plunger to the syringe tip and transferred to the sample vial for CE analysis. Often, only one exhalation (30  $\mu\text{L}$  of the sample) was enough to obtain a sizable amount of EBC for CE analysis. During the sweeping procedure, the condensate collected from the walls is naturally mixed and a representative EBC sample is thus obtained. To ensure the simplicity, low cost and wide availability of the consumables for EBC sampler, a saliva trap was not included. Therefore one can occasionally expect the interference from saliva droplets. This can happen especially when long-time sampling is applied or EBC is collected from patients from serious lung diseases and/or children. Nevertheless, saliva contamination is very rare and happened in every ca. 1 in 100 samples. Contaminated samples were easily identified and excluded from further investigation. *Fig.2* shows a CE separations of anions of two EBC samples; EBC sample obtained from 3 deep exhalations (=approx. 100  $\mu\text{L}$  EBC) and the same EBC sample spiked with 1  $\mu\text{L}$  of human saliva (to simulate the saliva contamination). The EBC sample spiked with saliva displays a huge increase of all anions and these concentration levels are significantly above the upper limit of concentrations found in healthy individuals or patients suffering from various lung diseases [9,10]. Another even more suitable indicator is the thiocyanate, normally present in human saliva [11], but not in EBC.



*Figure 2.* Anionic separation of EBC sample and EBC sample spiked with human saliva. BGE: 20 mM MES/HIS, 2 mM 18-crown-6, 30  $\mu\text{M}$  CTAB. HV: positive +15 kV, C4D detection. Injection: hydrodynamic for 30 s at height of 10 cm.



*Figure 3.* Cationic separation of EBC sample and EBC sample spiked with human saliva. BGE: 20 mM MES/HIS, 2 mM 18-crown-6, 30  $\mu\text{M}$  CTAB. HV: negative -15 kV, C4D detection. Injection: hydrodynamic for 30 s at height of 10 cm.

Cationic analysis of the same two samples (*Fig.3*) showed the highest increase of sodium and potassium, again high above the normal levels for healthy and patients concentrations. Other cations were also increased.



### 3.2. Day-to-day repeatability of ionic content of EBC

In the next part of the study, day-to-day repeatability of ionic content of EBC (5 following days) obtained from three healthy persons (#1, #2 and #3) was studied. Samples were collected in the morning, before breakfast and all volunteers were asked not to drink or eat before the sampling, the only exception was drinking of tap water. EBC samples were obtained from single exhalation by using the EBC sampler. All samples were analyzed by CE-C4D and peak areas of all ions in the electropherograms were integrated. Resulting peak areas (PA) for selected ions (ammonium, sodium, acetate and nitrite) were plotted vs. each of the five days (Fig. 4). From this figure, a sufficient repeatability of all ions present in EBC samples during five days can be seen. Although the spread might seem high, this is caused mainly by the physiological variability of the ion concentration in EBC.

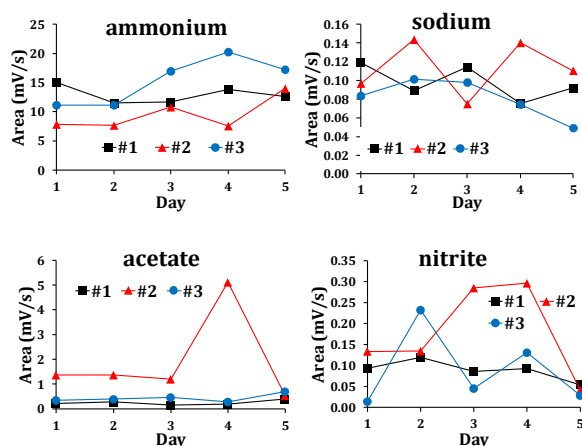


Figure 4. PA of selected ions found in EBC for a 5 day-to-day study. Separation and detection conditions as in Fig. 2 and 3.

### 3.3. Day-to-day repeatability of pH of EBC

Except for cations and anions which were measured by CE-C4D, other parameters e.g. exhaled breath volume (measured by spirometry) or pH of EBC can be necessary for standardization and diagnostic purposes [12,13]. pH of EBC samples was measured and the current experimental setup allowed pH measurements from as little as 10  $\mu$ L of

EBC. pH of EBC obtained from a single exhalation can thus be easily measured. pH of EBC samples obtained in the previous section (day-to-day repeatability) was measured before and after degassing with  $N_2$  (1.8 atm for 5 minutes), and resulting data can be seen in Fig. 5. It is known that degassing of liquids by an inert gas, such as  $N_2$ , can remove dissolved gases, e.g.  $CO_2$  [14]. Our experiments support this premise since we observed an increase of pH in all samples after degassing with  $N_2$ . Moreover, degassing the EBC samples significantly increased the repeatability of pH values during the five day-to-day study.

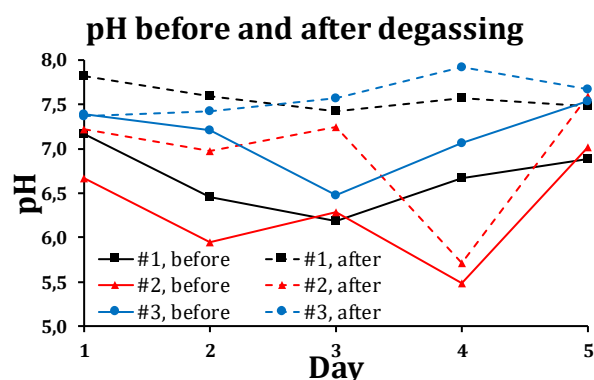


Figure 5. A five-day trend in pH of EBC sample obtained from three healthy volunteers. pH of EBC before (full line) and after degassing with  $N_2$  (dash line).

### 3.4. Other parameters influencing the ionic content of EBC and pH

The influence of various food or drinks on the ionic content and pH of EBC was also examined. Two EBC control samples (taken in the morning, before breakfast and only water was allowed to drink) were compared with the two EBC samples from the same day and after one of the following foods/drinks – breakfast, lunch, chocolate, coffee, orange juice, milk, chewing gum or after teeth cleaning with toothpaste or dentifrice. The time delay between each of the influences and sample collection was 10 minutes. In Fig. 6 and 7, examples of separation of anions and cations in EBC samples collected in the morning, after ingestion of orange juice and after chewing a chewing gum are shown. In overall, significant differences were found between samples and therefore at least one-hour

delay following flushing the mouth with DI water was suggested before the EBC collection to obtain a relevant EBC sample.

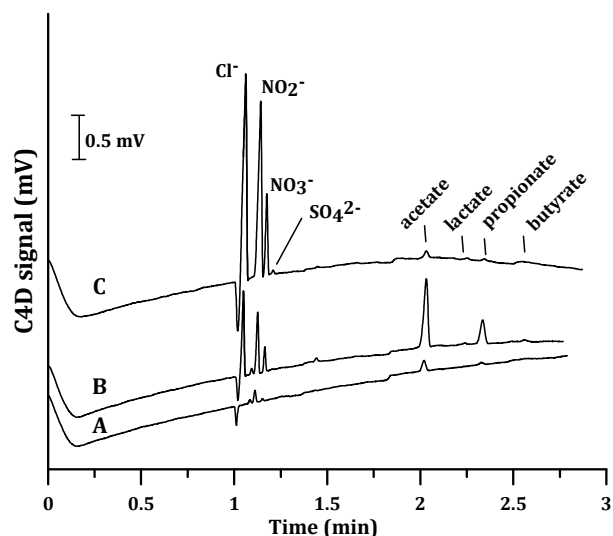


Figure 6. Separation of anions in different EBC samples. EBC sample collected in the morning (A), EBC sample after ingestion of orange juice (B) and after chewing a chewing gum (C). BGE: 20 mM MES/HIS, 2 mM 18-crown-6, 30  $\mu$ M CTAB. HV: positive +12.4 kV, C4D detection. Injection: hydrodynamic for 20 s at height of 12.5 cm.

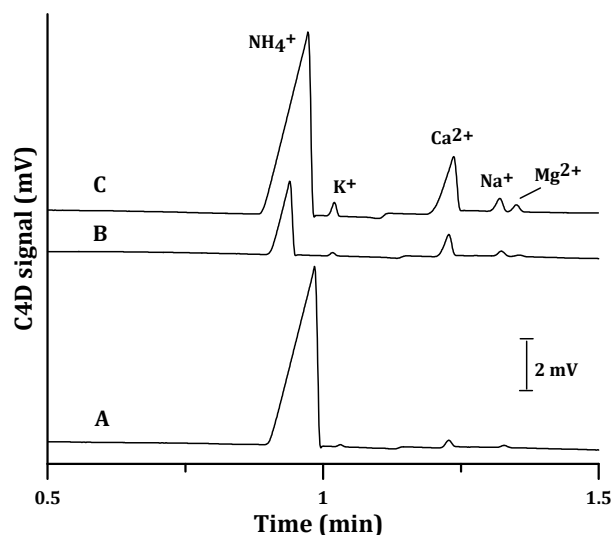


Figure 7. Separation of cations in different EBC samples. EBC sample collected in the morning (A), EBC sample after ingestion of orange juice (B) and after chewing a chewing gum (C). BGE: 20 mM MES/HIS, 2 mM 18-crown-6, 30  $\mu$ M CTAB. HV: negative -14.5 kV, C4D detection. Injection: hydrodynamic for 20 s at height of 12.5 cm.

## 4. Conclusions

In this work, capillary electrophoresis with contactless conductometric detection was used to study day-to-day repeatability of ionic content of EBC. EBC pH before and after degassing with a stream of  $N_2$  was studied by a pH-microelectrode during five consecutive days. Influence of various food and drinks on ionic content of EBC was investigated and showed significant differences between EBC samples. It has been shown that EBC is influenced by various parameters. Therefore, healthy individuals or patients should provide their EBC samples in the morning before breakfast/tooth brushing or at least one-hour after last food or drink followed flushing their mouth with DI water.

## SYMBOLS

CE	capillary electrophoresis
C4D	contactless conductivity detection
CTAB	cetyltrimethylammonium bromide
DI	deionized
EBC	exhaled breath condensate
HIS	histidine
ID	inner diameter
MES	2-(N-morpholino)ethanesulfonic acid
OD	outer diameter

## Acknowledgement

The authors acknowledge the financial support from the Ministry of Health of the Czech Republic (Grant No. 17-31945A). Part of this research was carried out under the project CEITEC 2020 (LQ1601) with financial support from the Ministry of Education, Youth and Sports of the Czech Republic under the National Sustainability Programme II and with institutional support RVO:68081715.

## REFERENCES

- [8] Shaheen, N. J.; Hansen, R. A.; Morgan, D. R.; Gangarosa, L. M.; Ringel, Y.; Thiny, M. T.; Russo, M. W.; Sandler, R. S.: The burden of gastrointestinal and liver diseases, *Am. J. Gastroenterol.*, 2006 **101**(9), 2128-2138 10.1111/j.1572-0241.2006.00723.x

- [9] Vakil, N.; van Zanten, S. V.; Kahrilas, P.; Dent, J.; Jones, R.: The Montreal definition and classification of gastroesophageal reflux disease: A global evidence-based consensus, *Am. J. Gastroenterol.*, 2006 **101** (8), 1900-1920 10.1111/j.1572-0241.2006.00630.x
- [10] Johnston, N.; Ondrey, F.; Rosen, R.; Hurley, B. P.; Gould, J.; Allen, J.; DelGaudio, J.; Altman, K. W.: Airway reflux, *Ann. NY. Acad. Sci.*, 2016 **1381**, 5-13 10.1111/nyas.13080
- [11] Emilsson, O. I.; Gislason, P.; Olin, A. C.; Janson, C.; Olafsson, I.: Biomarkers for Gastroesophageal Reflux in Respiratory Diseases, *Gastroenterol. Res. Pract.*, 2013, 9 10.1155/2013/148086
- [12] Shimizu, Y.; Dobashi, K.; Mori, M.: Exhaled breath marker in asthma patients with Gastroesophageal reflux disease, *J. Clin. Biochem. Nutr.*, 2007 **41** (3), 147-153.
- [13] Greguš, M.; Foret, F.; Kubáň, P.: Single-breath analysis using a novel simple sampler and capillary electrophoresis with contactless conductometric detection, *Electrophoresis*, 2015 **36**(4), 526-533 [10.1002/elps.201400456](https://doi.org/10.1002/elps.201400456)
- [14] Greguš, M.; Foret, F.; Kubáň, P.: Portable capillary electrophoresis instrument with contactless conductivity detection for on-site analysis of small volumes of biological fluids, *J. Chromatogr. A*, 2016 **1427**, 177-185 10.1016/j.chroma.2015.11.088
- [15] Almstrand, A.-C.; Bake, B.; Ljungström, E.; Larsson, P.; Bredberg, A.; Mirgorodskaya, E.; Olin, A.-C.: Effect of airway opening on production of exhaled particles, *J. Appl. Physiol.*, 2010 **108**(3), 584-588 [10.1152/jappphysiol.00873.2009](https://doi.org/10.1152/jappphysiol.00873.2009)
- [16] Greguš, M.; Foret, F.; Kindlová, D.; Pokojová, E.; Plutinský, M.; Doubková, M.; Merta, Z.; Binková, I.; Skříčková, J.; Kubáň, P.: Monitoring the ionic content of exhaled breath condensate in various respiratory diseases by capillary electrophoresis with contactless conductivity detection, *J. Breath. Res.*, 2015 **9**, 027017 10.1088/1752-7155/9/2/027107
- [17] Kubáň, P.; Foret, F.: Exhaled breath condensate: Determination of non-volatile compounds and their potential for clinical diagnosis and monitoring. A review, *Anal. Chim. Acta*, 2013 **805**, 1-18 10.1016/j.aca.2013.07.049
- [18] Vitali, L.; Fávere, V.T.; Micke, G.A.: A new method to determine biological sample volume by short end multiple injection capillary electrophoresis: Application in determination of nitrate and thiocyanate in human saliva, *J. Chromatogr. A*, 2011 **1218**(16), 2327-2333 10.1016/j.chroma.2011.02.035
- [19] Vaughan, J.; Ngamtrakulpanit, L.; Pajewski, T.N.; Turner, R.; Nguyen, T.-A.; Smith, A.; Urban, P.; Hom, S.; Gaston, B.; Hunt, J.: Exhaled breath condensate pH assays are not influenced by oral ammonia, *Thorax*, 2005 **60**(1), 27-31 10.1136/thx.2003.020602
- [20] Kostikas, K.; Papatheodorou, G.; Ganas, K.; Psathakis, K.; Panagou, P.; Loukides S.: pH in expired breath condensate of patients with inflammatory airway diseases, *Am. J. Respir. Crit. Care Med.*, 2002 **165**(10), 1364-1370 10.1164/rccm.200111-0680C
- [21] Ahmadzai, H.; Huang, S.; Hettiarachchi, R.; Lin, J.L.; Thomas, P.S.; Zhang, Q.: Exhaled breath condensate: a comprehensive update, *Clin. Chem. Lab. Med.*, 2013 **51**(7), 1343-1361 10.1515/cclm-2012-0593

## **P7 DETERMINATION OF THE OLIGOSACCHARIDE COMPOSITION IN WORT SAMPLES BY CAPILLARY ELECTROPHORESIS**

TAMÁS GÁBOR SZILÁGYI<sup>1,2</sup>, BEÁTA HEGYESNÉ VECSERI<sup>2</sup>, ZSUZSANNA KISS<sup>2</sup>, LÁSZLÓ HAJBA<sup>1,3</sup>,  
ANDRÁS GUTTMAN<sup>1,3</sup>

1 Horváth Csaba Memorial Institute for Bioanalytical Research, University of Debrecen, Research Center of Molecular Medicine, Debrecen

2 Faculty of Food Science, Department of Brewing and Distilling, Szent István University, Budapest

3 MTA-PE Translational Glycomics Research Group, Research Institute for Biomolecular and Chemical Engineering, University of Pannonia, Veszprem, Hungary

\*Correspondence: hajba@mukki.richem.hu

### **Abstract**

The aim of our work was to determine the oligosaccharide composition in different wort samples by capillary electrophoresis (CE) in order to monitor their change during the brewing process with different yeast types. CE separates oligosaccharide molecules with the same molecular mass based on their hydrodynamic volume to charge ratio. The concentration of fermentable and non-fermentable sugars were monitored by CE to observe the effect of two different type of yeasts, *Saccharomyces pastorianus* and *Saccharomyces ludwigii*. The former is prevalently used as it first ferments the monosaccharides, then the higher sugar oligomers such as maltose, maltotriose, etc., to ethanol. The latter, on the other hand, while fully ferments the monosaccharides, it practically does not ferment the oligosaccharides. Therefore, breweries use this latter yeast to produce beers with low alcohol content. The CE traces of the wort samples obtained during the fermentation processes by these two yeasts readily identified the oligosaccharide signatures with high resolution that could be used for decision making about the process in hand. The high resolving power and short analysis time of CE offered a very convenient way to analyze the oligosaccharide composition of these wort samples with high sensitivity and separated all carbohydrates of interest in a rapid automated fashion.

### **Acknowledgment**

The authors gratefully acknowledge the support of the Momentum (Lendület) grant # 97101 of the Hungarian Academy of Sciences (MTA-PE Translational Glycomics) and the National Research, Development and Innovation Office (NKFIH) (K 116263) grants of the Hungarian Government. This work was also supported by the BIONANO\_GINOP-2.3.2-15-2016-00017 project.

## P8 MULTISCALE MODELING OF RECTIFYING BIPOLAR NANOPORE

Z. HATÓ<sup>1</sup>, M. VALISKÓ<sup>1</sup>, T. KRISTÓF<sup>1</sup>, D. GILLESPIE<sup>2</sup>, D. BODA<sup>1,3</sup>

1 Department of Physical Chemistry, University of Pannonia, Veszprém, Hungary

2 Department of Molecular Biophysics and Physiology, Rush University Medical Center, Chicago, USA

3 Institute of Advanced Studies Kőszeg (iASK), Kőszeg, Hungary

### Abstract

As our devices are getting smaller and smaller multiscale modelling is getting more and more important. Nanopores in nanofluidic devices can function as the central working units and the behaviour of selectivity and rectification can be determined here also. The mentioned nanopores can be used as a heart of a sensor. In the presence of an analyte molecule the ionic current flowing through the pore is modulated, so the analyte molecule can be detected selectively if it is bound to the nanopore. (see Eszter Márai poster: Using nanopores as sensors: a computer simulation study)

We present computer simulation results for a rectifying bipolar nanopore on two modeling levels. In an all-atom model, we use explicit water to simulate ion transport directly with the Molecular Dynamics technique. We address an old paradox: how do reduced models, whose assumptions should break down in a nanoscale device, predict experimental data? In a reduced model, we use implicit water model and apply the Local Equilibrium Monte Carlo method together with the Nernst-Planck transport equation. This hybrid method makes the fast calculation of ion transport possible at the price of lost details. We show that the implicit-water model is an appropriate representation of the explicit-water model when we look at the system at the device (i.e., input vs. output) level.

We find profound differences between the two models looking at details of concentration and potential profiles that do not influence the basic behavior of the model as a device.

Our simulations show that reduced models can still capture the overall device physics correctly, even though they get some important aspects of the molecular-scale physics quite wrong; reduced models work because they include the physics that is necessary from the point of view of device function. Therefore, reduced models can suffice for general device understanding and device design, but more detailed models might be needed for molecular level understanding.

### References

- [1] Z. Ható et al, Multiscale modeling of a rectifying bipolar nanopore: explicit-water versus implicit-water simulations, *Phys.Chem.Chem.Phys.*, 2017, 19, 17816 doi: 10.1039/c7cp01819c

## **P9 CFD MODELLING BASED INVESTIGATION OF THE ELECTROOSMOTIC AND FORCED CONVECTION FLOW IN CAPILLARY ELECTROPHORESIS APPLICATIONS**

GÁBOR JÁRVÁS,<sup>1,2\*</sup> MÁRTON SZIGETI,<sup>1,2</sup> AND ANDRÁS GUTTMAN<sup>1,2,3</sup>

1 Horvath Csaba Memorial Institute of Bioanalytical Research, University of Debrecen, Hungary

2 MTA-PE Translational Glycomics Group, University of Pannonia, Veszprem, Hungary

3 SCIEX Separations, Brea, CA

\*Correspondence: jarvasg@gmail.com

### **Abstract**

Capillary Electrophoresis (CE) is a powerful liquid phase separation technique that plays an important role in multiple aspects of bioanalysis. Connection of CE to mass spectrometry via a sheathless electrospray interface represents a good combination of separation efficiency and detection versatility. However, using sheathless type MS interfaces, the electrospray process requires a bulk flow that can be accommodated either by electroosmotic pumping or forward pressurization. Simulation of the flow profiles in both instances is possible by Computational Fluid Dynamics (CFD) modeling. Electroosmotic Flow (EOF) is an electrokinetic phenomenon due to the formation of an electrical double layer (EDL) at the inner surface of the separation capillary columns upon the application of an electric field. EDL theory has been introduced as a continuum description of surface chemistry at the capillary column walls. CFD simulation of the EOF is challenging as it involves the following three different physical approaches. 1) The formed electric field is the function of ion concentration in the background electrolyte, thus mass transport is calculated; 2) The electric field is recovered from the gradients of the potential field; 3) The bulk fluid motion is described by the Navier-Stokes equations (including the continuity function). Based on the simulation results (calculated flow profiles) we compared the effect of forced convection by the applied pressure with and without EOF. Our simulation data was reduced into practice by electrokinetically introducing APTS labeled maltose into a capillary column and mobilized by 1) electrophoresis with no pressure applied but in the presence of EOF; 2) pressure driven flow with no electric field and consequently no EOF applied; 3) electrophoresis in the presence of EOF and pressure applied. The efficiencies (theoretical plate numbers) of the detected peaks were calculated from the resulting electropherograms and were approximately 500,000 plates/m for electrophoresis with EOF, 5000 plates/m for pressure only and 35,000 plates/m for electrophoresis with simultaneous EOF and pressure applied, backing up the simulation data.

### **Acknowledgment**

The authors gratefully acknowledge the support of the Momentum (Lendulet) grant # 97101 of the Hungarian Academy of Sciences (MTA-PE Translational Glycomics) and the National Research, Development and Innovation Office (NKFIH) (K 116263) grants of the Hungarian Government. This work was also supported by the BIONANO\_GINOP-2.3.2-15-2016-00017 project.

# P10 STUDY OF VARIOUS PARAMETERS INFLUENCING CONTENT OF EXHALED BREATH CONDENSATE WITH IMPORTANCE FOR DIAGNOSIS OF GASTROESOPHAGEAL REFLUX DISEASE

ZSUZSANNA KOVÁCS,<sup>1</sup> ANNA FARKAS,<sup>1</sup> AND ANDRÁS GUTTMAN<sup>1,2</sup>

<sup>1</sup> Horváth Csaba Laboratory of Bioseparation Sciences, Research Centre for Molecular Medicine, University of Debrecen, 98. Nagyerdei körút, 4032 Hungary

<sup>2</sup> MTA-PE Translational Glycomics Research Group, University of Pannonia, Veszprem, 10. Egyetem utca, 8200 Hungary

**Abstract** – Multiple myeloma (MM) is an inoperable malignancy of human plasma cells. Its pathogenesis is poorly understood, and mounting evidence indicates that the bone marrow microenvironment of tumor cells has a prominent role in the malignant process [1]. It was shown that in multiple myeloma (MM) IgG paraproteins exhibit unique oligosaccharide profiles [2] (Figure 1). In this paper we report on a novel approach for paraprotein N-glycosylation analysis from intact and papain digested IgGs. Capillary Electrophoresis with Laser Induced Fluorescence detection (CE-LIF) was used for the analysis of the glycan patterns.

**Keywords:** Multiple myeloma, N-glycan, CE-LIF

## 1. Introduction

Multiple myeloma (MM) is characterized as the clonal proliferation of malignant plasma cells and practically incurable even in the 21st century. Its diagnosis can be established upon the latest IMWG criteria that can be time-consuming because of the deceptive clinical symptoms. The most frequently used processes to diagnose multiple myeloma utilize particular combinations of laboratory, imaging, and bone marrow biopsy test results as criteria. To widen the diagnostic toolsets and to help finding proper remedies, all modern omics methods have been recently attempted. As a result, several new and unexpected oncogenic mechanisms were discovered influencing the mutations of the genes involved in the translation mechanisms, histone methylation, blood coagulation, and paraprotein formation. These latter ones are abnormal immunoglobulin fragments or immunoglobulin light chains (both kappa and lambda) and their appearance in the serum indicates the presence of a proliferating clone of immunoglobulin-producing cells. In the serum of healthy individuals, the albumin to immunoglobulin ratio is  $> 2$  and the total kappa ( $\kappa$ ) to lambda ( $\lambda$ ) chain ratio is approximately 3:1. Any changes of this ratio can be an indication of blood cell dyscrasia or multiple myeloma. Indeed, in MM the albumin to immunoglobulin ratio can be  $< 1$  as a result of the extra paraproteins in the bloodstream. More importantly in respect to this study, paraprotein glycosylation can be indicative of the progress of the disease. Analysis of

the N-glycosylation profiles of paraproteins may have prognostic value for multiple myeloma patient treatment. The most frequently used N-glycan analysis methods are LC, CE, NMR, and MS. While all of these methods can be utilized and have their own advantages, in this paper we used CE-LIF detection for rapid and high resolution N-glycan profiling of the samples.

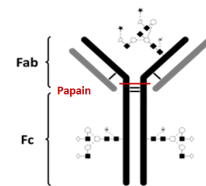


Figure 1. Papain mediated cleavage of the IgG heavy chains.

## 2. Experimental

### 2.1. Samples and Measurements

**Serum samples:** Serum samples were collected at the Clinical Centre of Internal Medicine (University of Debrecen, Hungary) from six untreated, six treated, and six remission multiple myeloma patients as well as six male and six female healthy controls using clot activator containing serum tubes (BD, Franklin Lakes, NJ). The collected blood samples were centrifuged at  $7500 \times g$  for 30 min and the serum fractions were stored at  $-70^\circ\text{C}$  until further processing.

Correspondence: andras.guttman@hlbs.org

Papain digestion: One milliliter papain solution was mixed with 4 mL of 0.2M EDTA and 0.2M cysteine containing 1 x PBS buffer (pH 6.5). Hundred microliters of this reagent was added to 100  $\mu$ L of serum sample and incubated at 37°C for 4 h. The reaction was stopped by the addition of 20  $\mu$ L iodoacetamide solution (0.3 M in 1 x PBS).

Partitioning of the Fc fragment: 200  $\mu$ L of the papain digest was used for all sample types (controls, untreated, treated, and remission stage multiple myeloma patients) after purification on 20  $\mu$ L bed-volume Protein A affinity microcolumns. 200  $\mu$ L of 1 x capture buffer (0.7 M NaH<sub>2</sub>PO<sub>4</sub>, 0.7 M NaCl, pH 7.4) was added to the serum samples and then the resin was washed with 200  $\mu$ L of 0.7 M NaH<sub>2</sub>PO<sub>4</sub> 0.7 M NaCl buffer (pH 7.4). Then 200  $\mu$ L of 140mM NaCl was added to the microcolumns and the Fc fragments were eluted with 200  $\mu$ L of 15% acetic acid. The samples were transferred to 10 kDa spin-filters and centrifuged at 11 270 x g for 10 min. The N-glycan moiety of the partitioned Fc fractions was PNGase F digested in situ on the filter and the liberated N-glycans were APTS labeled as described below.

$\kappa/\lambda$  light chain partitioning: 200  $\mu$ L of the digestion mixture was used for all samples and purified on CaptureSelect LC-kappa (Hu) and CaptureSelect LC-lambda (Hu) filled affinity microcolumns. 200  $\mu$ L of capture buffer (1 x PBS) was added to the serum samples and then the resin was washed with 200  $\mu$ L of 1 x wash buffer I (0.7 M NaH<sub>2</sub>PO<sub>4</sub> 0.7M NaCl pH7.4). Then 200  $\mu$ L of 1 x wash buffer II (140 mM NaCl) was added to the microcolumns and the IgG kappa/lambda light chains were eluted with 200  $\mu$ L of 0.1 M glycine-HCl (pH 2.0). The samples were then transferred to 10 kDa centrifugal filters, centrifuged at 11 270 x g for 10 min. The prepared light chain fractions were PNGase F treated in situ on the filter and APTS labeled as described below.

N-Glycan release and fluorophore labeling: One microliter of serum from each patient samples were diluted by 10  $\mu$ L of HPLC grade water then 1  $\mu$ L of denaturing buffer was added followed by incubation at 65°C for 10 min. The reaction mixture was filtered through 10 kDa spin-filters at 11 270 x g for 10 min. Both the denatured serum samples were digested in situ on the filters by the addition of 29  $\mu$ L of NaHCO<sub>3</sub> buffer (20 mM, pH 7.0) and 1  $\mu$ L

of PNGase F, followed by incubation at 37°C overnight. The released N-glycans were centrifuged through 10 kDa spinfilters at 7500 x g for 10 min and dried in SpeedVac prior to the fluorophore labeling step. 6  $\mu$ L of 20mM 8-aminopyrene-1,3,6-trisulfonic acid in 15% acetic acid and 2  $\mu$ L of NaCNBH<sub>3</sub> (1 M in THF) were added to the dry pellet and incubated at 37°C overnight. The labeled samples were purified with CleanSeq magnetic beads.

Capillary electrophoresis: A P/ACE MDQ System was used to perform all capillary electrophoresis analyses. The separations were monitored by LIF detection using a 488 nm Ar-ion laser with a 520 nm emission filter. Fifty centimeters effective length (60 cm total) 50  $\mu$ m id NCHO capillaries were employed with the NCHO separation gel buffer system for the analysis. The 32 Karat software was used for data acquisition and processing.

### 3. Results and Analysis

#### 3.1. Experiments

After specifying the main peaks of interest in the healthy sample, the global serum N-glycan profiles of the three patient groups of untreated freshly diagnosed (trace B), treated (trace C), and remission stage (trace D) patients were analyzed and a

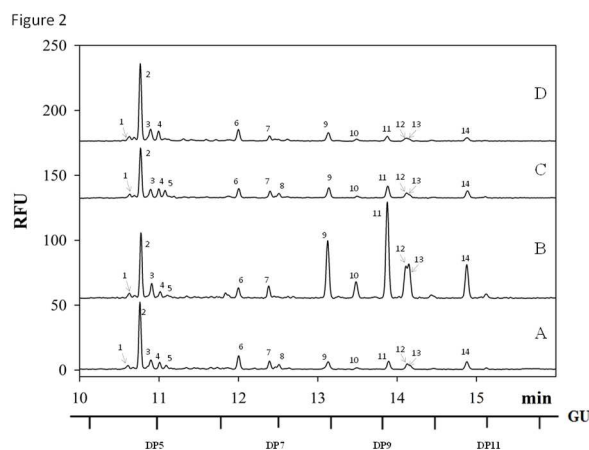
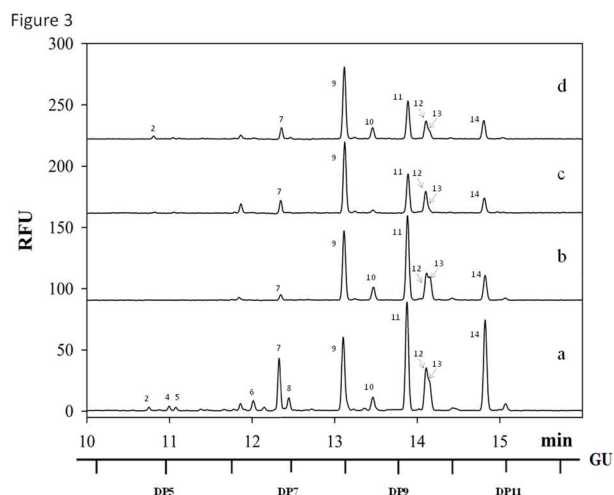


Figure 2. Comparison of the CE-LIF traces of APTS-labeled serum N-glycomes of healthy control (A), freshly diagnosed untreated (B), treated (C), and remission stage (D) multiple myeloma patients. Conditions: NCHO capillary with 50 cm effective length (total length 60 cm, 50  $\mu$ m id); NCHO gel buffer; Separation temperature: 25°C; Voltage: 30.0 kV (0.17 min ramp) reversed polarity; Injection: 1.0 psi, 0.5 s.

representative set of results are compared in the traces in Figure 2.



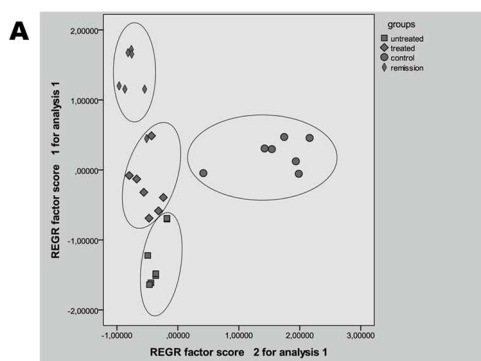
As one can observe, the traces revealed major differences in peak distribution. Trace B (freshly diagnosed untreated patient group) show significant changes in the sialoform (peaks 1–8) to neutral (peaks 9–14) carbohydrate ratio. Next, we narrowed our focus to study the N-glycosylation of the Fc chain fractions that were partitioned by Protein A specific microcolumn affinity pulldowns after papain digestion. It is well known that in the serum



**Figure 3.** N-linked carbohydrate profiles of the IgG Fc fractions from healthy control (a), freshly diagnosed untreated (b), treated (c), and remission stage (d) multiple myeloma patients. Conditions were the same as in Fig. 2. Injection: 1.0 psi, 0.5 s.

N-glycome, most neutral species are originated from the approximately 25% immunoglobulin fraction.

Fig. 3 shows, the changes in the N-linked glycan profile of the immunoglobulin Fc fractions were not as substantial as was observed in the global serum N-glycan profiles (Fig. 1), except the peak ratio

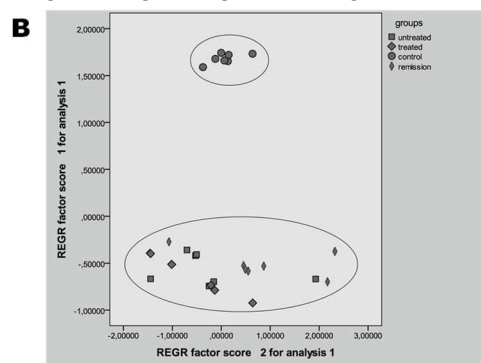


**Figure 4A.** Principal component analysis of the N-glycosylation data obtained from the analysis of the total serum (A).

changes in comparison to the sialoforms (peaks 1–

8) in which case all three patient sample traces showed significantly increased neutral glycan species (peaks 9–14).

During the statistical analysis, first the total serum N-glycosylation data was evaluated. In this case, the first two principal component axes accounted for 47.3 and 17.2% data variance, respectively, representing 64.5% of data variance cumulatively, which was sufficient to resolve the data into four distinct groups as shown in Fig. 4A (control, untreated, treated, and remission) [3]. In case of the papain digested IgG fragments (Fig. 4B), the first



**Figure 4B.** Principal component analysis of the N-glycosylation data obtained from the analysis of the papain partitioned IgG chains (B).

two principal component axes accounted for 45.6 and 16.1% data variance, respectively, representing 61.7% of data variance cumulatively, which was sufficient only to distinguish the control group from the MM patients, but not within the latter ones.

#### 4. Discussion

Structural characterization of the N-glycan profiles of healthy control and multiple myeloma patients of freshly diagnosed untreated, treated, and remission stage has been reported. The study included the N-glycosylation analysis of human serum plasma proteins at the global level as well as the Fc fractions of papain digested paraproteins. Principal component analysis clearly differentiated the four patient groups on the basis of total serum N-glycosylation analysis. The N-glycan patterns of the papain digested immunoglobulin fragments on the other hand, did not show such distinctive differences, and only discriminated between the healthy control and the patients. Based on our results, we suggest that in addition to the higher information content of the total serum level N-

glycosylation pattern analysis for multiple myeloma patients, it is also much easier to accomplish, i.e. does not require additional digestion and partitioning steps, thus easier to implement in clinical settings.

## 5. Conclusion

This paper reports on glycosylation pattern characterization of normal as well as untreated, treated and remission MM patient plasma samples. Comparison of the control total serum and control Protein A partitioned IgG glycans with untreated, treated and remission MM patient revealed subtle differences. Based on these preliminary results, the next step will be validation of these potential glycan biomarkers in a larger cohort.

## SYMBOLS

$\kappa$ : immunoglobulin G kappa chain

$\lambda$ : immunoglobulin G lambda chain

CE-LIF: Capillary Electrophoresis with Laser Induced Fluorescence detection

APTS: 8-aminopyrene-1,3,6-trisulfonic acid

## Acknowledgement

The authors acknowledge the support of the National Research, Development and Innovation Office (NKFIH) (K 116263) grants of the Hungarian Government. This work was also supported by the Hungarian Ministry of Human Capacities and the BIONANO\_GINOP-2.3.2- 15-2016-00017 project.



SUPPORTED BY THE ÚNKP-17-3-IV-DE-236 NEW NATIONAL EXCELLENCE PROGRAM OF THE MINISTRY OF HUMAN CAPACITIES

## REFERENCES

- [1] Chapman, M.A.; Lawrence, M.S.; Keats, J.J.; Cibulskis, K. et al.: Initial genome sequencing and analysis of multiple myeloma. *Nature*, 2011 Mar 24;471(7339):467-72. DOI: 10.1038/nature09837
- [2] Farooq, M.; Takahashi, N.; Arrol, H.; Drayson, M.; Jefferis, R.: Glycosylation of polyclonal and paraprotein IgG in multiple myeloma. *Glycoconjugate Journal*, 1997 Jun;14(4):489-92. DOI: 10.1023/A:1018555619519
- [3] Kovacs, Z.; Simon, A.; Szabo, Z.; Nagy, Z.; Varoczy, L.; Pal, I.; Csanky, E.; Guttman A.: Capillary electrophoresis analysis of N-glycosylation changes of serum paraproteins in multiple myeloma. *Electrophoresis*, 2017 Sep;38(17):2115-2123. DOI: 10.1002/elps.201700006. Epub 2017 Feb 21.

# P11 BIOBANKING OF PATIENT SAMPLES FOR GENO – GLYCOMIC LUNG DISEASE BIOMARKER STUDIES

RENÁTA KUN<sup>1\*</sup>, ESZTER CSÁNKY<sup>2</sup>, MIKLÓS SZABÓ<sup>2</sup>, ZSOLT RÓNAI<sup>3</sup>, ZSUZSANNA KOVACS<sup>1</sup>, MÁRTON SZIGETI<sup>4</sup>, GÁBOR JÁRVÁS<sup>4</sup>, LÁSZLÓ HAJBA<sup>4</sup>, BOGLÁRKA DÖNCZŐ<sup>1</sup>, ANDRÁS GUTTMAN<sup>1,4</sup>

1 Horváth Csaba Laboratory of Bioseparation Sciences, University of Debrecen, Hungary

2 Semmelweis Hospital, Miskolc, Hungary

3 Department of Medical Chemistry, Semmelweis University, Budapest, Hungary

4 MTA-PE Translational Glycomics Group, University of Pannonia, Veszprem, Hungary

\*E-mail: kunrenata86@gmail.com

Abstract – Comprehensive geno – glycomic studies to identify new tumor biomarkers depend on appropriate collection and registration of biological materials, included tissues, blood samples or other body fluids from a large number of patients and healthy individuals with associated well- documented clinical information from the sample donors [1].

**Keywords:** biobanking, data collecting, glycans, genomic, lung diseases, CE-LIF

## 1. Introduction

The aim of the project is biological sample collection (biobanking), which involves a group of lung cancer (LC), COPD and LC+COPD patients, as well as healthy controls with the necessary TUKEB permission and informed patient consent.

## 2. Experimental

The sample groups consist of approximately 300 LC (adenocarcinoma), 300 COPD, 300 LC+COPD, as well as 300 non – cancer related control samples. One of the most important part of our biobanking workflow is the clinical data collection from each patient (age, smoking habits, occupation, morbidity and co-morbidity, tumor/COPD stage, histology, TNM chemotherapies, surgeries, etc.) from MedWorkS information system and fill out the relevant questionnaires. The collected samples are stored at -80 °C until processed.

## 3. Results

The high number of patient samples (biobank) will allow thorough geno - glycomic characterization of the four patient classes with high statistical value

including SNP study, N-glycan site specificity analysis as well as quantitation of glycan linkage and positional isomers at the specific sites.

## 4. Discussion

In summary, this novel combination of genetic and glycomic approaches will provide comprehensive information for both sides of the problem in hand and will lead to the identification of disease specific glycosylation (site and microheterogeneity) changes of acute phase proteins and similarly reveal changes in glycosylation of some known cancer biomarkers.

## Acknowledgement

The authors acknowledge the support of the MTA-PE Translation Glycomics Grant (#97101) and NKFIH (#K116263) grants of the Hungarian Government.

## REFERENCES

- [1] Malm, J., et al., *Developments in biobanking workflow standardization providing sample integrity and stability.* J Proteomics, 2013. **95**: p. 38-45.

# P12 SINGLE-CELL ANALYSIS OF ACTIVE CASPASE 3/7 IN APOPTOTIC CELLS

VOJTĚCH LEDVINA<sup>1,2</sup> AND KAREL KLEPÁRNÍK<sup>1\*</sup>

1 Institute of Analytical Chemistry of the CAS, v. v. i., Brno, 602 00 Czech Republic

2 Faculty of Science, Masaryk University, Brno, 601 77 Czech Republic

**Abstract** – Caspases are proteases that play key role in the process of apoptosis, the programmed cell death. Among them, caspase-3 and -7 are main executioner caspases that cleave many vital proteins during apoptosis and after their widespread activation, the process cannot be reversed. To analyze caspase-3/7 activation within single cells, a miniaturized device for parallel analysis of eight samples was developed. The LOD suitable for detection of active caspase-3/7 in both apoptotic and non apoptotic cells was reached.

**Keywords:** single-cell analysis, caspase detection, bioluminescence

## 1. Introduction

For the detection of active caspase-3/7, we used a commercially available Caspase-Glo<sup>®</sup> 3/7 reagent (Promega), a homogeneous BL assay based on modified luciferin-luciferase system developed by O'Brien et al. [1]. In this assay, aminoluciferin is conjugated with caspase-3/7 specific tetrapeptide Asp-Glu-Val-Asp which makes the substrate inaccessible for luciferase. After the peptide is cleaved by the caspase, free aminoluciferin is immediately oxidized by firefly luciferase in presence of Mg<sup>2+</sup>, ATP and oxygen, resulting in a photon emission. Due to the enzymatic nature of the assay, single caspase molecule generates multiple photons and the background signal is very low due to the absence of interfering autofluorescence, photobleaching and excitation light. Presented will be a miniaturized device for parallel BL detection of active caspase-3/7 content in single apoptotic and non-apoptotic cells that is compatible with analyses of suspended cells selected and transferred by a micromanipulator mounted on a microscope. Our objective was to reach LOD below the average amount of active caspase-3/7 in a single apoptotic cell.

## 2. Experimental

Micromass cultures isolated from mouse forelimbs at embryonic day 12 were selected as a model. Two experimental groups were prepared: a control group cultivated in standard culture medium (non-

apoptotic cells) and an experimental group treated with 5 μM camptothecin for 8 hours (apoptotic cells). From one to six cells were collected at a time and transferred into individual detection microvials using ICSI micromanipulator (Eppendorf) with a 20 μm o.d. holding pipette for *in vitro* fertilization, mounted on an inverted microscope Olympus IX71. The microvials were made from glass capillaries of sizes i.d./o.d. 1.3/1.9 mm and filled with 4 μl of Caspase-Glo<sup>®</sup> 3/7 reagent. After filling, all eight microvials were placed into a stainless steel revolving carousel vial holder and the whole assembly was mounted in front of the PMT detection window. The BL emission leaving individual vials was detected consecutively and repeatedly in 5min intervals for a period of about 80 minutes until the steady state was reached in all vials. The detection assembly consisted of a photon counting head H7421-40 series with a heat sink and fan A7423, counting unit C8855-01 and power supply unit with temperature control M 9011 (Hamamatsu Photonics, Japan). The photon counting head is furnished with a PMT having a GaAsP/GaAs photocathode and a thermoelectric cooler. The cooler maintains PMT at a constant temperature of 0 °C and reduces thermal noise generated by the photocathode, thus increasing S/N ratio.

## 3. Results and Analysis

The background signal of Caspase-Glo<sup>®</sup> 3/7 reagent linearly increases with its volume as the amount of accidentally released aminoluciferin accessible for

---

\*Correspondence: klep@iach.cz

luciferase oxidation rises. On the other hand, if the volume is too low, modified luciferin can be depleted before the BL reaches its steady state and the signal can be decreased. To reach the signal maximum, we used detection microvials of 1.3 mm i.d. filled with 4  $\mu$ l of the reagent. This was enough to provide sufficient substrate delivery to the active caspase-3/7 molecules while maintaining relatively low background signal. Further decrease of the background was achieved by using a PMT with cooled photocathode. The photocathode yields a dark current of only about 3-6 photon counts/s which is very low compared to the blank signal of the reagent (about 62 counts per second).

The device was tested with apoptotic and non-apoptotic cells. In both cases, the caspase-3/7 content linearly increased with the number of cells. The average signal intensity for apoptotic cells ranged from 157-952 counts/s and was approximately three times higher than for the non-apoptotic cells where the signal ranged from 56-241 counts/s.

The LOD and LOQ were calculated as three and ten times the standard deviation of a blank, respectively. For apoptotic cells the LOD/LOQ were evaluated to be 0.27/0.86 of a caspase-3/7 content in an average apoptotic cell and for the non-apoptotic cells 0.46/2.92 of a caspase-3/7 content in an average non-apoptotic cell. The device is therefore suitable for both detection and quantification of active caspase-3/7 in individual apoptotic cells. In the case of non-apoptotic cells, caspase-3/7 can be reliably detected but not quantified.

#### 4. Discussion

High standard deviation were apparent particularly in the case of apoptotic cells. The deviations can be

caused by cell heterogeneity, by the aspects of dynamics of camptothecin induced caspase activation in individual cells and the effect of the cell cycle phase of individual cells. It is also possible that camptothecin may not diffuse properly to the lower layers of cells in Petri dishes that adhere directly to the surface during cultivation. Some very low activation of caspase-3/7 in non-apoptotic cells can be due to the *ex vivo* approach and micromass treatment. It may also suggest some physiological basal level of activation or can even point to possible non-apoptotic functions of these caspases.

#### 5. Conclusion

We have developed a miniaturized device for sensitive detection of caspase-3/7 activity within single cells. The LOD reached is suitable for detection in both apoptotic and non-apoptotic cells but reliable quantification is so far possible only in apoptotic cells. Although the throughput of our device is limited, it could be in principle increased by using a microplate with 1536 wells each filled with 3–10  $\mu$ l of the reagent, providing a sensitive PMT detector monitoring each well separately without any crosstalk and fast and effective system for cell collection and transfer."

#### Acknowledgement

The research was supported by the Grant Agency of the Czech Republic, project no. 17-01995s. The work was also supported by the Institute of Analytical Chemistry of the CAS under the Institutional Research Plan RVO: 68081715.

#### REFERENCES

- [1] O'Brien M. A., Daily W. J., Hesselberth P. E., Moravec R. A., Scurria M. A., Klaubert D. H., Bulleit R. F., Wood K. V. *J Biomol Screen*, 2005, 10 (2), 137-148.

# P13 APPLICATION OF AMINO ACIDS AND PEPTIDES FOR OLIGOSACCHARIDE LABELING

JANA KRENKOVA,\* JAN PARTYKA, AND FRANTISEK FORET

Department Bioanalytical Instrumentation, Institute of Analytical Chemistry of the Czech Academy of Sciences, Veveří 97, Brno, 60200, CZECH REPUBLIC

Abstract – In this work, we report on application of positively charged compounds such as basic amino acids (Arg, Lys, His) and peptides (2Gly6His, 6His, 5Gly3His) for oligosaccharide/*N*-linked glycan labeling followed by CE-MS analysis.

**Keywords:** oligosaccharides, labeling, CE-MS

## 1. Introduction

Glycosylation of proteins is the most common post-translational modification, which strongly influences biological activity and function of proteins [1]. Traditional glycosylation and glycan analysis involves multistep, multiday sample preparation. However, derivatization/labeling seems to be a critical step in the glycan analysis by either LC or CE separation with fluorescence/mass spectrometric detection. Labeling has several purposes: (a) enhancing sensitivity of analysis with different detectors (fluorescence or MS), (b) an increase in hydrophobicity of the highly hydrophilic oligosaccharides resulting in increased chromatographic retention in the reversed-phase LC mode, or (c) introducing a charge into the neutral oligosaccharide molecules effecting the electrophoretic mobility.

Various labels have been used for glycan labeling [2]. The most widely used tags are 2-aminobenzamide (2-AB), 2-aminobenzoic acid (2-AA), 2-aminopyridine (PA), 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), and 8-aminopyrene-1,3,6-trisulfonic acid (APTS). The majority of labeling procedure for CE separation has been performed using a negatively charged fluorophore APTS followed by CE with laser-induced fluorescence (CE/LIF) and/or mass spectrometry (CE-MS) detection. However, an attachment of a cationic label with a high number of positive charges should provide faster migration in CE, simplify selection of separation buffers for electrophoretic sample concentration and allow more sensitive detection by MS in a positive ion mode.

## 2. Experimental

All the chemicals used in this study were purchased from Sigma Aldrich (Prague, Czech Republic). The separation capillary was obtained from Polymicro Technologies (Phoenix, AZ, USA).

Oligosaccharides were dissolved in a solution containing water, methanol and acetic acid. A 10-fold excess of labels (amino acids or peptides) were added. A solution of sodium cyanoborohydride in methanol was added and the mixture was incubated at 55°C for 20 hours. Afterward, the solvents were evaporated in a speed-vac concentrator and the samples were re-dissolved in water before CE-MS analysis.

CE-MS experiments were conducted on a 7100 CE system (Agilent Technologies, Waldbronn, Germany) connected to the maXis Impact mass spectrometer (ESI/TOF-MS) (Bruker Daltonics, Bremen, Germany) using a polyimide based liquid junction CE-MS interface (*Fig.1*). The CE separations were performed in the fused silica capillaries (uncoated or LPAA coated). Solutions of 1 mol/L formic acid or 100 mmol/L acetic acid were used as background electrolytes and spray liquids. The MS detection was performed in the positive ion mode. The detail experimental conditions are shown in the figure captions.

---

\*\*\*\*Correspondence: krenkova@iach.cz

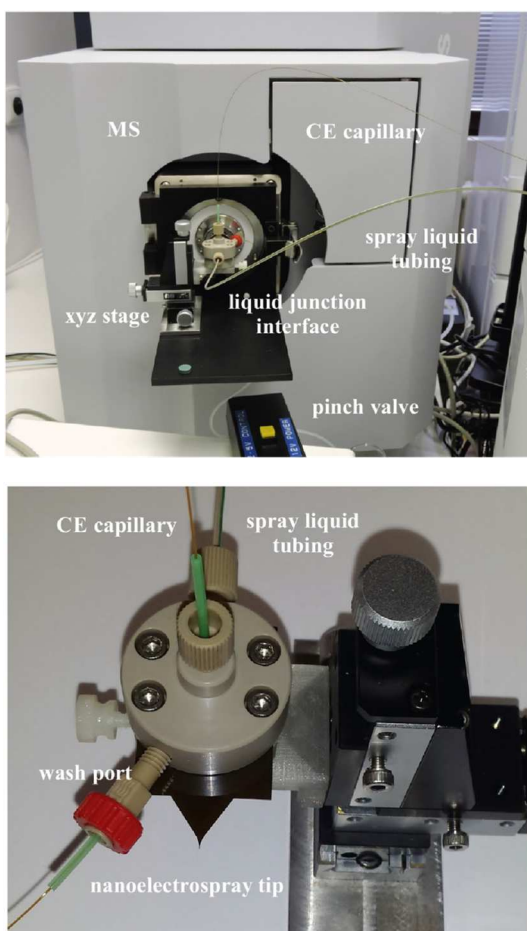


Figure 1. Polyimide based liquid junction CE-MS

### 3. Results and Discussion

In this study, we have tested various basic amino acids (Arg, Lys, His) as well as peptides (2Gly6His, 6His, 5Gly3His) for oligosaccharide labeling suitable for following CE-MS analysis in the positive ion mode. The standard oligosaccharides and *N*-linked glycans released from bovine ribonuclease B or fetuin were labeled by reductive amination. A scheme is shown in Fig.2. A 10-fold excess of labels was added to the oligosaccharides in order to form a Schiff base, which was subsequently reduced by sodium cyanoborohydride to the corresponding aminodeoxyalditol.

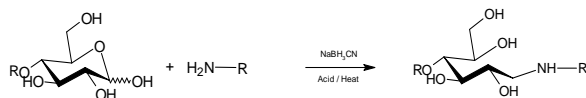


Figure 2. Oligosaccharide labeling by reductive amination.

All the selected amino acids and peptides provide a positive charge of labeled oligosaccharides in the background electrolytes and spray liquids (acetic acid or formic acid) leading to sufficient separation by capillary electrophoresis. The migration times of labeled standard oligosaccharides (isomaltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose) were different depending on a number of charges provided by the label as shown in Fig.3. The highest velocity showed oligosaccharides labeled by a hexahistidine peptide (2Gly6His). Maltooligosaccharides labeled by a hexahistidine sequence migrated approximately two times faster than the same standards labeled by histidine alone.

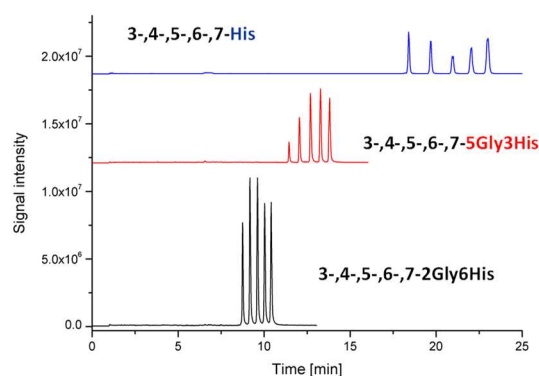


Figure 3. CE-MS analysis of maltooligosaccharides labeled by different number of histidines. Experimental conditions: CZE - 50  $\mu\text{m}$  ID capillary, 70 cm, uncoated, BGE: 1 mol/L formic acid, 20 kV, hydrodynamic injection: 50 mbar, 10 s. MS - spray liquid: 1 mol/L formic acid; 0.5 atm, 1.7 kV, 1 Hz scan.

We have tested labeling of oligosaccharides by a hexahistidine peptide with and without a spacer, which was added to the sequence in order to minimize the steric hindrance and charge repulsion. We have observed only small difference in migration times of 2Gly6His- and 6His-labeled oligosaccharides. The oligosaccharide standards labeled by the 2Gly6His peptide migrated slightly slower than the standards labeled by the 6His peptide since two molecules of glycine make the oligosaccharide molecule larger without bringing any additional positive charge.

The labeling of more complex samples such as *N*-linked glycans released by peptide-*N*-glycosidase F from ribonuclease B and fetuin has been also tested. The labeling by a peptide 2Gly6His allows fast and efficient separation by capillary electrophoresis. Due to the multi-positively charged peptide the acidic *N*-linked glycans containing a various number of sialic acid in the molecules were detected as cations in the positive ion MS mode (Fig.4).

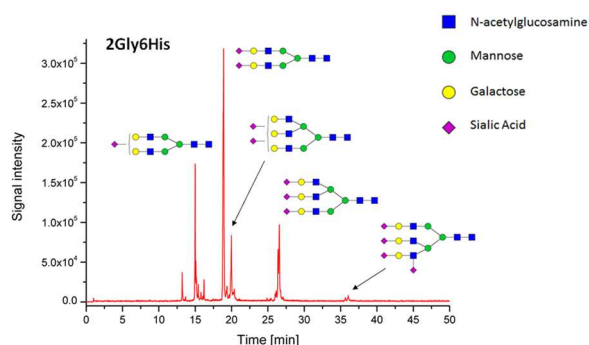


Figure 4. CE-MS analysis of fetuin *N*-linked glycans labeled by 2Gly6His peptide. Experimental conditions: CZE - 75  $\mu$ m ID capillary, 75 cm, LPAA coating, BGE: 100 mmol/L acetic acid, 20 kV, hydrodynamic injection: 50 mbar, 10 s. MS - spray liquid: 100 mmol/L acetic acid, 0.5 atm, 1.7 kV, 1 Hz scan.

#### 4. Conclusion

The basic amino acids and peptides have been used for oligosaccharide labeling via reductive amination. Fast and efficient CE separation and

sensitive MS detection of maltooligosaccharide standards have been achieved. The labeling by the multi-positively charged peptide (2Gly6His) has also enabled MS detection of both neutral and acidic *N*-linked glycans in the positive ion mode.

#### Acknowledgement

The research was supported by the Grant Agency of the Czech Republic (16-09283Y, P206/12/G014) and the institutional research plan (RVO: 68081715) of the Institute of Analytical Chemistry of the Czech Academy of Sciences.

#### REFERENCES

- [1] Spiro, R.G.: Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds, *Glycobiology*, 2002 **12**(4), 43R–56R DOI [10.1093/glycob/12.4.43R](https://doi.org/10.1093/glycob/12.4.43R)
- [2] Ruhaak, L.R., Zauner, G., Huhn, C., Bruggink, C., Deelder, A.M., Wuhrer, M.: Glycan labeling strategies and their use in identification and quantification, *Anal. Bioanal. Chem.* 2010 **397**(8), 3457-3481 DOI [10.1007/s00216-010-3532-z](https://doi.org/10.1007/s00216-010-3532-z)



# P14 CAPILLARY ELECTROPHORETIC ANALYSIS OF EXHALED BREATH CONDENSATE IN THE DIAGNOSIS OF GASTROESOPHAGEAL REFLUX DISEASE

PETR KUBÁŇ,<sup>1\*</sup> PAVOL ĎURČ,<sup>1</sup> JÚLIA LAČNÁ,<sup>1</sup> MICHAL GREGUŠ,<sup>1</sup> FRANTIŠEK FORET,<sup>1</sup> JIŘÍ DOLINA,<sup>2</sup> ŠTEFAN KONEČNÝ,<sup>2</sup> MARTINA DOUBKOVÁ,<sup>3</sup> DAGMAR KINDLOVÁ,<sup>3</sup> EVA POKOJOVÁ,<sup>3</sup> AND JANA SKŘIČKOVÁ<sup>3</sup>

1 Department of Bioanalytical Instrumentation, CEITEC Masaryk University, Veveří 97, Brno, 602 00, CZECH REPUBLIC

2 Internal Gastroenterology Department, University Hospital Brno, Faculty of Medicine, Masaryk University, Jihlavská 20, Brno, 625 00, CZECH REPUBLIC

3 Department of Respiratory Diseases and TB, University Hospital Brno, Faculty of Medicine, Masaryk University, Jihlavská 20, Brno, 625 00, CZECH REPUBLIC

**Abstract** – In this work, capillary electrophoresis with contactless conductometric detection was used for the analysis of ionic content of exhaled breath condensate to differentiate among the healthy individuals and patients with gastroesophageal reflux disease. The exhaled breath condensate was collected using a miniature sample collection device and the content was analyzed using a separation electrolyte composed of 20 mM 2-(N-morpholino) ethanesulfonic acid, 20 mM L-histidine, 2 mM 18-crown-6 and 30  $\mu$ M cetyltrimethylammonium bromide. The separation of anions took less than 2.5 minutes, while the cations were separated in less than 1.5 minutes. The most significantly elevated ions in the group of patients with gastroesophageal reflux were chloride, nitrate, propionate and butyrate. Although the number of subjects was too small to draw definite conclusions on discriminatory power of these ions, the pilot data hold promise for EBC as a useful non-invasive alternative to other methods used in gastroesophageal reflux disease diagnosis.

**Keywords:** capillary electrophoresis, exhaled breath condensate, ionic analysis, diagnosis, gastroesophageal reflux disease.

## 1. Introduction

Capillary electrophoresis is an attractive separation technique that is particularly suitable for the analysis of biological samples. Non-invasive sampling and analysis of alternative biofluids are gaining scientific attention, as these samples are acquired easily, can be obtained repeatedly without any particular stress to the patient and often have a simple matrix. One such a sample is exhaled breath condensate (EBC).

EBC is obtained by cooling the exhaled breath using a suitable cooling equipment and apparatus. EBC is the aqueous part of the exhaled breath that contains mainly condensed water from the breath and volatile compounds soluble in water, but also small respiratory droplets that carry the information on the lung condition, inflammation or oxidative/nitrosative stress. EBC was first used as a diagnostic sample by Sidorenko in the 1980s [1] and since then numerous articles on EBC have been published, including some recent reviews [2,3]. EBC is attractive not only to study one's lung condition [4,5], but may also find other applications, for instance in the assessment of inflammatory condition of trachea and esophagus. The latter is of particular importance, because gastroesophageal reflux disease (GERD) is one of

the most common diseases of the western world. Between 20 and 30% of general population in Europe and USA will suffer from GERD [6] and about 60% of the adult population will experience some type of GERD during their life span. GERD is often diagnosed using invasive instruments, such as 24-hour multichannel intraluminal impedance and pH monitoring (MII-pH) [7], during which the patient is required to continuously wear a narrow catheter in the esophagus. This device measures the spreading and volume of gas, liquid, and solid through the esophagus and evaluates the composition of refluxate. The data are eventually used to evaluate whether the person is suffering from the disease based on the number of recorded reflux episodes and DeMeester score [8] describing the severity of reflux. The use of the MII-pH is however rather unpleasant and up to 1/3 of subjects report discomfort. In this contribution, we have attempted to replace the above mentioned MII-pH measurement with significantly less invasive analysis of EBC. EBC was collected using a specially designed miniature sampler [4,9,10] that was previously developed in our laboratory. EBC samples were collected from several healthy volunteers and a group of patients suffering from symptoms of gastroesophageal reflux disease. Capillary electrophoresis was used for the analysis

\*\*\*Correspondence: Dr.Petr Kubáň,

petr.kuban@gmail.com

of ionic content of EBC samples and differences in selected ion concentrations were found between the groups.

## 2. Experimental

### 2.1. Instrumentation

An in-house built CE instrument was used for all electrophoretic separations at  $\pm 15$  kV using a high voltage power supply unit (Spellman CZE2000R Start Spellman, Pulborough, UK). Two Pt wires (0.5 mm outer diameter (OD), 3 cm length, Advent Research Materials Ltd., Eynsham, England) were inserted in the electrolyte vials to serve as electrodes. Fused-silica (FS) capillaries (50  $\mu\text{m}$  inner diameter (ID), 375  $\mu\text{m}$  OD, 39 cm total length, Polymicro Technologies, Phoenix, AZ, USA) were used for the separation. All CE experiments were performed at ambient temperature. A custom made contactless conductometric detector (Version 5.06, ADMET s.r.o., Prague, Czech Republic) operating at a frequency of 1.8432 MHz and voltage 50 V<sub>p-p</sub> was used for the detection of the separated analytes. Data were collected using an ORCA 2800 24-bit A/D converter and ECOMAC software ver. 0.254 (ECOM spol s.r.o., Praha, Czech Republic). In all experiments, hydrodynamic injection was applied to the samples, consisting of elevation of the sample vial to the height of 10 cm for 20 s.

The sampler for collection of EBC was developed in our group earlier [9]. Briefly, it was constructed from a 2 ml syringe (B. Braun Melsungen AG, Melsungen, Germany) cooled by a 5 cm long hollow aluminum cylinder of 2.5 cm OD with ID precisely matching the OD of the syringe (see Fig. 1).

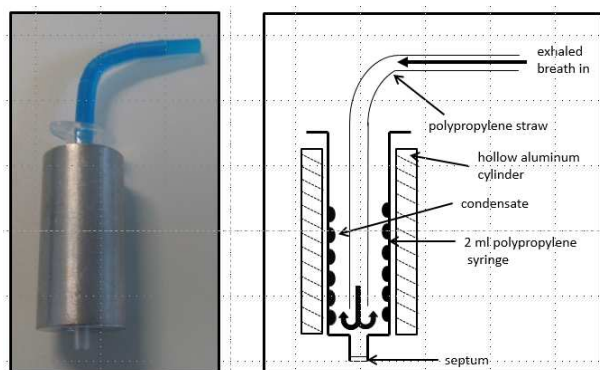


Figure 1. The photograph and scheme of the EBC sampler used in this work.

The cylinder was kept in dry ice at  $-80^\circ\text{C}$  prior to EBC collection and an insulation sleeve was used during EBC collection. A straw with OD of 6 mm, and wall thickness 0.2 mm (purchased in a local store) was used to exhale the air through the sampler. The end of the syringe was enclosed with a parafilm septum to avoid EBC loss.

### 2.2. Chemicals

All chemicals were of reagent grade (Sigma-Aldrich, Steinheim, Germany) and deionized (DI) water (Purite, Neptune, Watrex, Prague, Czech Republic) was used for stock solution preparation and dilutions. Background electrolyte (BGE) for CE measurements was prepared daily by diluting 100 mM stock solutions of 2-(N-morpholino)ethanesulfonic acid (MES), L-histidine (HIS) and 18-crown-6 to the required concentration. Cetyltrimethylammonium bromide (CTAB) was prepared as 10 mM stock solution in 5 % acetonitrile.

The separation electrolyte that was used for separation and quantitation of inorganic anions, cations and organic acids, consisted of 20 mM MES, 20 mM HIS, 2 mM 18-crown-6 and 30  $\mu\text{M}$  CTAB.

### 2.3. Capillary conditioning procedure

Prior to the first use, the separation capillary was preconditioned by flushing with 0.1 M NaOH for 30 min, DI water for 30 min and BGE solution for 10 min. Between two successive injections, the capillary was flushed with BGE solution for 1 min. At the end of a working day, the capillaries were washed with DI water for 15 min, followed by applying a vacuum for 5 min and stored dry overnight.

### 2.4. Sample collection procedure

The EBC was collected in the morning, the subjects did not eat or drink prior to EBC collection. The EBC was collected using the previously described EBC-sampler. The person was asked to deeply exhale 3-5 times into the sampler, but depending on the person's lung capacity, the number of exhalations was modified to collect approximately

100  $\mu\text{L}$  of the EBC. After the EBC sampling was completed, the condensate from the syringe walls was swept with the plunger to the syringe tip and transferred to the sample vial for CE analysis. Part of the EBC sample was also used in another experiment, published elsewhere, in which the pH was measured using a microelectrode.

### 2.5. Patients and healthy individual selection

In this initial screening, a group of healthy individuals and a group of patients diagnosed with asthma and pulmonary fibrosis were selected. The healthy individuals had no documented GERD, no heartburn and were free from other related symptoms. The healthy individuals were selected from approximately the same age group as the patients (i.e. the average age of 48 for healthy vs. the average age of 56 for the patients). The patients suffered from asthma and pulmonary fibrosis and were from the Department of Pulmonary Diseases of University Hospital in Brno.

## 3. Results and Analysis

### 3.1. Electrolyte selection

For separation of ionic content of EBC, the BGE composed of MES and HIS was chosen, as this electrolyte allows analysis of anions, organic acids and cations with good sensitivity. MES and HIS fulfill the criteria for suitable co- and counter-ion in CE separation using the contactless conductivity detection (C4D). The conductance of the respective BGE components is low, thus analytes including small organic acids (acetate, lactate, butyrate) will be detected as positive peaks. The addition of 18-crown-6 to the separation electrolyte improves the resolution of  $\text{K}^+$  and  $\text{NH}_4^+$  cations and does not influence the selectivity of other measured cations to a great extent, nor does it influence the separation of anions and organic acids. CTAB is added to the separation electrolyte to decrease the electroosmotic flow (EOF).

It should be noted here that although this separation electrolyte allows simultaneous separation of anions and cations using dual-opposite end injection [11], in this work anions and cations were determined separately, by switching the high

voltage polarity. Fig. 2 shows the separation of a model mixture of 8 anions that was achieved in less than 2.5 min.

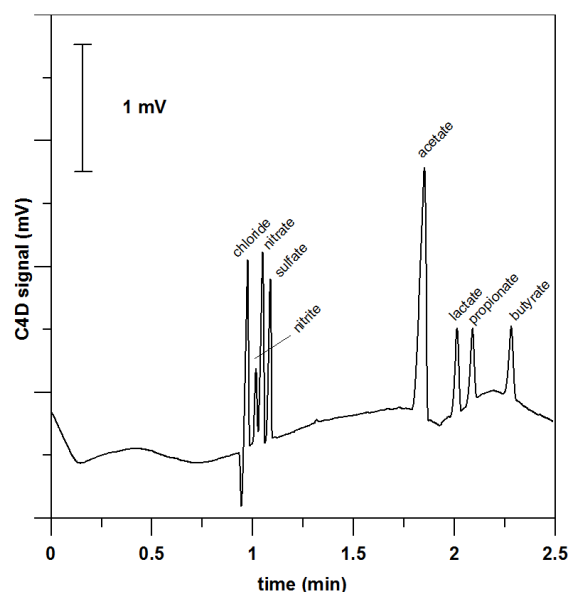


Figure 2. Separation of a model mixture of 4 inorganic anions and 4 organic acids. BGE: 20 mM MES/HIS, 2 mM 18-crown-6, 30  $\mu\text{M}$  CTAB. HV: positive +15 kV, C4D detection. Ion concentrations in the parentheses in  $\mu\text{M}$ : acetate (200); chloride, nitrate, lactate, propionate, butyrate (25); nitrite, sulfate (10).

The ion concentrations were selected to be similar to those found in EBC samples. Fig. 3 shows the separation of cations using the same conditions but reversed voltage polarity. The ammonium cation is usually present at 100-fold higher concentrations than other cations in the EBC, its concentration was thus also increased to 1000  $\mu\text{M}$  to reflect the expected concentration. Note that even at this high excess the separation of  $\text{NH}_4^+$  from  $\text{K}^+$  is sufficient and the BGE composition can be used for real sample analysis.

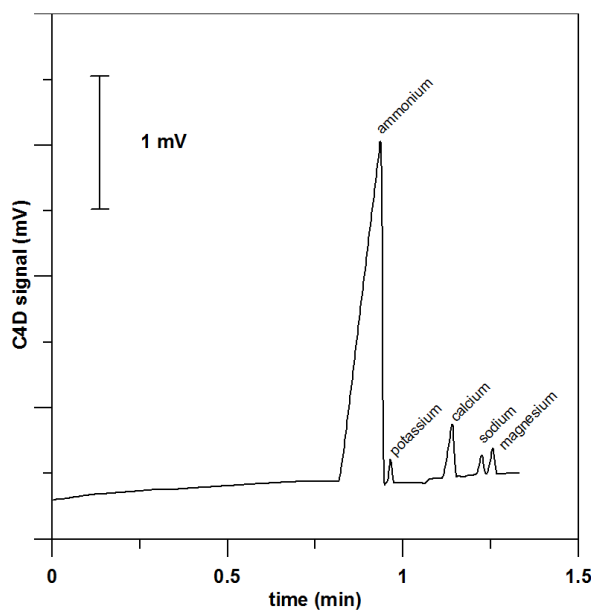


Figure 3. Separation of a model mixture of 5 inorganic cations. BGE: 20 mM MES/HIS, 2 mM 18-crown-6, 30  $\mu$ M CTAB. HV: negative -15 kV, C4D detection. Ion concentrations in the parentheses in  $\mu$ M: ammonium (1000); calcium (25), potassium, sodium and magnesium (10).

### 3.2. Analysis of anions in EBC samples

From each subject, the EBC sample was collected as described earlier. About 50  $\mu$ L of the sample was transferred into an Eppendorf vial and hydrodynamically injected into the CE system. The EBC samples were analyzed first for anions and organic acids, using positive HV polarity and then for cations using negative HV polarity, with the HV electrode placed in the detection vial. The concentration of all 13 ions in the samples was evaluated using calibration curves measured with standard solutions, the parameters, such as the calibration equation, regression coefficient ( $R^2$ ) and concentration range, are shown in Table 1. In this initial screening we have attempted to analyze several samples from the healthy group and patients that were diagnosed as having the acid or weakly acid reflux and identify the ions that would be significantly different in these groups.

Table 1. Calibration equations of all separated anions, cations and organic acids. BGE: 20 mM MES/HIS, 2 mM 18-crown-6, 30  $\mu$ M CTAB. HV: positive +15 kV or negative -15 kV, C4D detection.

Ion	Calibration equation	$R^2$	range ( $\mu$ M)
$\text{Cl}^-$	$y=0.0278x + 0.0258$	0.9957	0.6-25
$\text{NO}_2^-$	$y=0.0270x + 0.0023$	0.9994	0.6-25
$\text{NO}_3^-$	$y=0.0315x + 0.0166$	0.9987	0.6-25
$\text{SO}_4^{2-}$	$y=0.0656x + 0.0143$	0.9980	0.25-10
acetate	$y=0.0156x + 0.0200$	0.9995	1-200
lactate	$y=0.0207x + 0.0097$	0.9996	0.6-25
propionate	$y=0.0189x + 0.0024$	0.9983	0.6-25
butyrate	$y=0.0188x - 0.0001$	0.9990	0.6-25
$\text{NH}_4^+$	$y=0.0211x - 0.1437$	0.9993	25-1000
$\text{K}^+$	$y=0.0347x + 0.0013$	0.9994	0.25-10
$\text{Ca}^{2+}$	$y=0.0528x + 0.0059$	0.9995	0.6-25
$\text{Na}^+$	$y=0.0356x + 0.0079$	0.9996	0.25-10
$\text{Mg}^{2+}$	$y=0.0618x - 0.0054$	0.9995	0.25-10

Fig. 4 shows series of electropherograms of anions and organic acids in a healthy individual, patient with acid reflux and patient with weakly acid reflux. One can clearly see that the concentrations of several anions, most notably chloride, nitrate and organic acids are significantly higher in the patient group than in the healthy volunteer. Some small inorganic anions such as nitrite and nitrate are often found in the EBC of patients with respiratory diseases as markers of the nitrosative stress. On the other hand, the presence of an increased concentration of chloride and organic acids may be significant towards the GERD diagnosis.

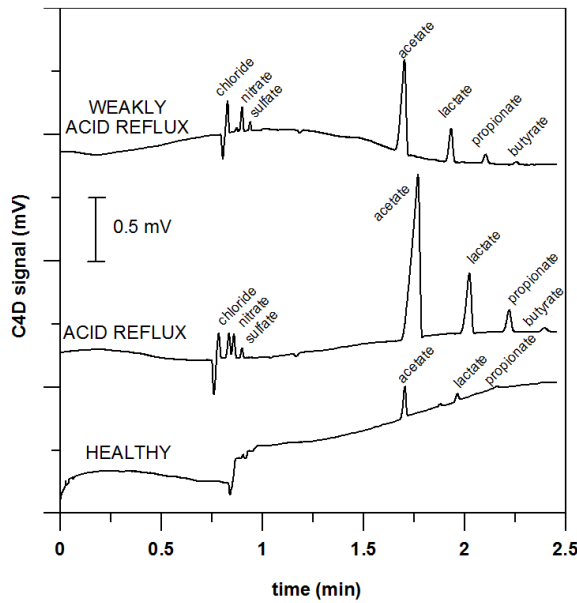


Figure 4. Separation of anions in EBC samples of healthy person, person with acid reflux and person with weakly acid reflux. BGE: 20 mM MES/HIS, 2 mM 18-crown-6, 30  $\mu$ M CTAB. HV: positive +15 kV, C4D detection.

### 3.3. Analysis of cations in EBC samples

The analysis of cations in the same samples can be seen in Fig. 5. Although higher concentrations of ammonia cation were found in patients compared to healthy individual, the content was not significantly different from the healthy individual, and this applied for other cations ( $K^+$ ,  $Na^+$ ,  $Ca^{2+}$ ,  $Mg^{2+}$ ) as well.

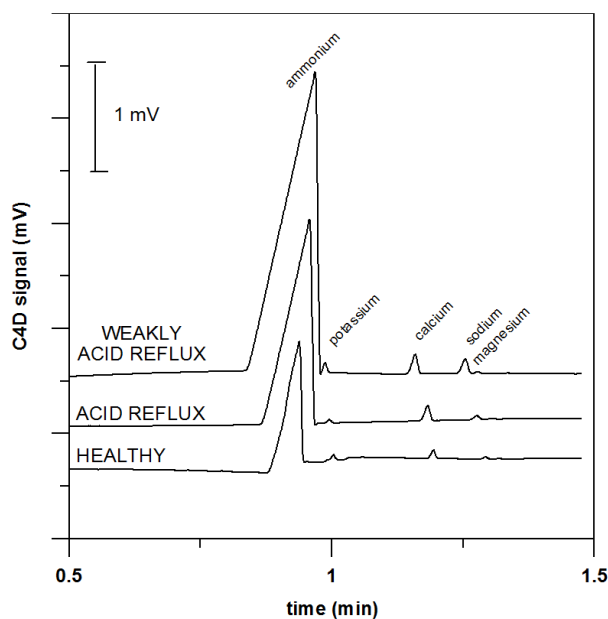


Figure 5. Separation of cations in EBC samples of healthy person, person with acid reflux and person with weakly acid

reflux. BGE: 20 mM MES/HIS, 2 mM 18-crown-6, 30  $\mu$ M CTAB. HV: negative -15 kV, C4D detection.

## 4. Discussion

We have tested a limited number of subjects and attempted to establish, whether a simple EBC sample could be used as a surrogate for more invasive, expensive and tedious diagnostic methods. The analysis of anions showed some promising results with regards to selected small inorganic anions (chloride and nitrate) and organic acids that were elevated in the patients suffering from GERD symptoms, compared to healthy volunteers. Unfortunately in the cation analysis, the differences were not significant and cations were deemed unsuitable.

## 5. Conclusion

We used capillary electrophoretic analysis of EBC for the first time in an attempt to distinguish the groups of patients with GERD from healthy individuals. CE with C4D was used for analysis of small inorganic anions, cations and organic acids present in the EBC samples. Although it has been shown that selected samples had elevated concentrations of chloride, nitrate, butyrate and propionate, the number of subjects was too small to draw definite conclusions on discriminatory power of these ions. It also seems that although there is a difference between healthy and acid reflux/weakly acid reflux groups, the results between acid and weakly acid reflux are very similar and these two groups cannot be separated based on the current data. A larger scale clinical study, in which EBC of patients and healthy individuals is scrutinized with regard to ion content, pH and concentration of other biomarkers (for instance pepsin in saliva) to obtain statistically significant results for evaluation is being currently performed. Nevertheless, the initial data hold some promise for EBC as a useful non-invasive alternative to other methods used in GERD diagnostics.

## SYMBOLS

BGE	background electrolyte
C4D	contactless conductivity detection
CE	capillary electrophoresis
CTAB	cetyltrimethylammonium bromide

DI	deionized
EBC	exhaled breath condensate
GERD	gastroesophageal reflux disease
HIS	L-histidine
ID	inner diameter
MES	2-(N-morpholino)ethanesulfonic acid
OD	outer diameter

## Acknowledgement

The research was supported by the grant from the Ministry of Health of the Czech Republic, grant no. 17- 31945A.

## REFERENCES

- [1] Sidorenko, G.I.; Zborovskii, E.I.; Levina, D.I.: Surface-active properties of the exhaled air condensates (a new method of studying lung function), *Ter. Arkh.*, 1980 **52**, 65-68
- [2] Kubáň, P.; Foret, F.: Exhaled breath condensate: Determination of non-volatile compounds and their potential for clinical diagnosis and monitoring. A review, *Anal. Chim. Acta* 2013 **805**, 1-18 10.1016/j.aca.2013.07.049
- [3] Hayes, S.A.; Haefliger, S.; Harris, B.; Pavlakis, N.; Clarke, S.J.; Molloy, M.P.; Howell W.M.: Exhaled breath condensate for lung cancer protein analysis: a review of methods and biomarkers, *J. Breath Res.*, 2016 **10**(3), 034001 10.1088/1752-7155/10/3/034001
- [4] Greguš, M.; Foret, F.; Kindlová, D.; Pokojová, E.; Plutinský, M.; Doubková, M.; Merta, Z.; Binková, I.; Skříčková, J.; Kubáň, P.: Monitoring the ionic content of exhaled breath condensate in various respiratory diseases by capillary electrophoresis with contactless conductivity detection, *J. Breath. Res.*, 2015 **9**, 027017 10.1088/1752-7155/9/2/027107
- [5] Aldakheel, F.M.; Thomas, P.S.; Bourke, J.E.; Matheson, M.C.; Dharmage, S.C.; Lowe, A.J.: Relationships between adult asthma and oxidative stress markers and pH in exhaled breath condensate: a systematic review, *Allergy*, 2016 **71**(6), 741-757 10.1111/all.12865
- [6] El-Serag, H.B.; H.B.; Sweet, S.; Winchester, C.C.; Dent, J.: Update on the epidemiology of gastro-oesophageal reflux disease: a systematic review, *Gut.*, 2014 **63**(6), 871-880 [10.1136/gutjnl-2012-304269](https://doi.org/10.1136/gutjnl-2012-304269)
- [7] Tutuian, R.; Castell, D.O.: Review Article: complete gastro-oesophageal reflux monitoring-combined pH and impedance, *Aliment. Pharmacol. Ther.*, 2006 **2**, 27-37 10.1111/j.1365-2036.2006.03039.x
- [8] Johnson, L.F.; Demeester T.R.: Twenty-four-hour pH monitoring of the distal esophagus. A quantitative measure of gastroesophageal reflux, *Am. J. Gastroenterol.*, 1974 **62**, 325-332
- [9] Greguš, M.; Foret, F.; Kubáň, P.: Single-breath analysis using a novel simple sampler and capillary electrophoresis with contactless conductometric detection, *Electrophoresis*, 2015 **36**(4), 526-533 [10.1002/elps.201400456](https://doi.org/10.1002/elps.201400456)
- [10] Greguš, M.; Foret, F.; Kubáň, P.: Portable capillary electrophoresis instrument with contactless conductivity detection for on-site analysis of small volumes of biological fluids, *J. Chromatogr. A*, 2016 **14**(27), 177-185 10.1016/j.chroma.2015.11.088
- [11] Kuban, P.; Karlberg, B.; Kuban, P.; Kuban, V.: Application of a contactless conductometric detector for the simultaneous determination of small anions and cations by capillary electrophoresis with dual-opposite end injection, *J. Chromatogr. A*, 2002 **964**, 227-241 10.1016/S0021-9673(02)00656-8

# P15 MULTISCALE MODELING OF RECTIFYING BIPOLAR NANOPORE

ESZTER MÁDAI<sup>1</sup>, MÓNIKA VALISKÓ<sup>1</sup> AND DEZSŐ BODA<sup>1,2</sup>

<sup>1</sup> Department of Physical Chemistry, University of Pannonia, Veszprém, Hungary

<sup>2</sup> Institute of Advanced Studies Kőszeg (iASK), Kőszeg, Hungary

## Abstract

For nanopores, there can be made a difference between natural and artificial nanopores, as ion channels and carbon nanotubes or nanopores cauterized into foils. Artificial nanopores have microscopic-length and a nanoscopic radius-size. Ion transport through the nanopore is connected with an important property: the selectivity. Due to this property the nanopore can be used, for instance, as a nanoscopic porous sensor.

The basic concept of our model nanopore sensor is that the presence of an analyte molecule modulates the ionic current flowing through the pore. The analyte molecule (usually a biomolecule) is detected selectively if it is bound to the nanopore. If the binding is a reversible process involving physical forces that are weaker than chemical bonds, the probability that the analyte molecule binds to the nanopore is proportional to its concentration. From calibration curves (current vs. concentration) the analyte concentration can be determined.

In this modeling work, we create simple models the membrane, the pore and the electrolyte. Negative charges are located on the cylindrical pore's wall to make the pore cation selective. For the electrolyte, the Primitive Model of electrolytes is used, which treats the ions as hard spheres with their charges located in the centers, and the solvent as a constant dielectric continuum. The binding sites are modeled with a square-well potential. We applied the Local Equilibrium Monte Carlo (LEMC) method coupled with Nernst-Planck (NP) equation. This method (NP+LEMC) is suitable for study even micromolar concentrations.

We present a modeling study to analyze the device sensitivity (defined as  $I/I_0$ , where  $I$  and  $I_0$  are the currents in the presence and absence of the analyte, respectively) to the various model parameters: number, strength, and width of the binding sites; properties of the electrolyte and the analyte. Through the calculated concentration profiles, the molecular mechanisms of the system can be understood.

## P16 QUANTITATIVE ASSESSMENT AND AUTOMATIC ID OF GLYCANS OF CQA IMPORTANCE IN BIOLOGICS DEVELOPMENT

ANDRAS GUTTMAN<sup>1,2</sup>, MARTON SZIGETI<sup>2,3</sup>, BRIGITTA MESZAROS<sup>2</sup>, JEFF CHAPMAN<sup>1</sup>

1 Sciex, Brea, CA, USA

2 Horváth Csaba Laboratory of Bioseparation Sciences, University of Debrecen, Hungary;

3 MTA-PE Translational Glycomics Research Group, Research Institute for Biomolecular and Chemical Engineering, University of Pannonia, Veszprem, Hungary

### Abstract

Carbohydrate profiling of the conserved asparagine linked glycosylation site (Asn 297 in most instances) of monoclonal antibody therapeutics is a key step in each phase of the product development cycle in the biopharmaceutical industry. Efficacy, stability, half-life and immunogenicity are all highly dependent on the N-glycosylation of protein therapeutics. The two major effector functions N-glycosylation may affect in therapeutic monoclonal antibodies are antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), via alterations in core fucosylation and antennary galactosylation, respectively [1]. The presence of high mannose type glycans affects half-life via interaction with mannose-binding lectins (MBL). Therefore, accurate and quantitative analysis of mAb N-glycosylation provides important information to meet the glycosylation aspects of critical quality attributes (CQA) of biologics development. Capillary electrophoresis with laser induced fluorescent detection (CE-LIF) is one of the highest resolution methods to separate fluorophore (APTS) labeled oligosaccharides. CE-LIF with the recently introduced triple internal standard based automated GU calculation method in conjunction with instant structural assignment of glycans of CQA importance using the built-in database provided qualitative and quantitative glycan identification with special focus on core fucosylation (ADCC, CQA), antennary galactosylation (CDC, CQA) and the presence of high mannose structures (serum half-life, CQA) [2].

### References

[1] Natsume, A., R. Niwa, and M. Satoh, Improving effector functions of antibodies for cancer treatment: Enhancing ADCC and CDC, *Drug Design Development and Therapy*, 2008, **3**, 7-16.

[2] Karalis, V.D., From Bioequivalence to Biosimilarity: The Rise of a Novel Regulatory Framework, *Drug Research*, 2016, **66**, 1-6.

### Acknowledgment

The authors gratefully acknowledge the support of the Momentum (Lendület) grant # 97101 of the Hungarian Academy of Sciences (MTA-PE Translational Glycomics) and the National Research, Development and Innovation Office (NKFIH) (K 116263) grants of the Hungarian Government. This work was also supported by the BIONANO\_GINOP-2.3.2-15-2016-00017 project.



# P17 DEVELOPMENT OF AN UHPLC-MS/MS METHOD FOR ANALYSIS OF A NOVEL CARDIOPROTECTIVE AGENT JAS 2 AND ITS METABOLITE

HANA PISKÁČKOVÁ,<sup>1\*</sup> PETRA REIMEROVÁ,<sup>1</sup> JAROSLAV ROH,<sup>2</sup> JANA SEDLÁKOVÁ<sup>2</sup> AND PETRA ŠTĚRBOVÁ KOVAŘÍKOVÁ<sup>1</sup>

1 Department of Pharmaceutical Chemistry and Pharmaceutical Analysis, Faculty of Pharmacy in Hradec Králové, Charles University, Akademika Heyrovského 1203 Hradec Králové, 500 05, CZECH REPUBLIC

2 Department of Organic And Bioorganic Chemistry, Faculty of Pharmacy in Hradec Králové, Charles University, Akademika Heyrovského 1203 Hradec Králové, 500 05, CZECH REPUBLIC

**Abstract** – The exact cause of anthracycline induced cardiotoxicity remains unknown. Dexrazoxane is the only approved drug for myocardium protection. However, the lack of information regarding mechanism of action and its structure-activity relationship hinders development of new, more effective and safer cardioprotective drugs. The aim of this project is to develop a pilot UHPLC-MS/MS method for determination of a novel analogue of dexrazoxane, JAS-2 and its metabolite in cell culture medium. Developed conditions will be further optimized for analyses of JAS-2 and its metabolite in different relevant biological materials (e.g. plasma, heart tissue, etc) and applied to a bioactivation study *in vitro* and *in vivo*.

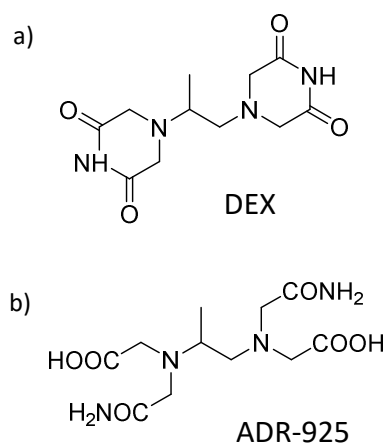
**Keywords:** UHPLC-MS, Dexrazoxane, Cardioprotective drugs

## 1. Introduction

Anthracycline chemotherapeutics (doxorubicine, daunorubicine) are one of the most effective anticancer drugs clinically used in the therapy of hematologic malignancies and several solid tumors (e.g. breast, ovarian and gastric carcinoma, bone sarcoma, etc.) [1, 2]. However, their clinical use is limited by the incidence of cardiotoxicity, especially its chronic forms, which lead to the development of congestive heart failure [2-4]. The exact cause of anthracycline induced cardiotoxicity remains unknown. It is assumed that these drugs are able to form reactive oxygen species that further produce extremely toxic and reactive hydroxyl radicals and this reaction is catalyzed by iron (Fe) ions. Another hypothesis attributes an important role to the interaction of anthracyclines with topoisomerase II $\beta$  [1, 4].

Dexrazoxane (DEX, Fig 1a) is the only approved drug effective in preventing anthracycline induced cardiotoxicity. DEX was traditionally assumed to be a prodrug and its cardioprotective effect was attributed to the Fe chelation properties of its metabolite ADR-925 (Fig 1b). Meanwhile, recent investigations suggest that cardioprotective effect of DEX is more complex and may be associated with the interaction of the parent drug with topoisomerase II $\beta$  [3, 5, 6]. The lack of information about the mechanism of cardioprotective effect of DEX hinders development of novel, more effective and safer analogues.

4,4'-(butane-2,3-diyl)bis(piperazine-2,6-dione) (JAS-2, Fig 2a) was synthesized as novel analogue of DEX. Pilot studies performed on neonatal rat cardiomyocytes indicate that JAS-2 is more effective in protection of cardiomyocytes from toxic effect of anthracyclines as compared with DEX. The further development of this novel analogue requires a modern



**Figure 1.** The only approved cardioprotective drug preventing anthracycline induced cardiotoxicity – dexrazoxane (a) and its metabolite (b) with chelating properties.

Correspondence: [piskacha@faf.cuni.cz](mailto:piskacha@faf.cuni.cz)

Table 1: Overview of SRM for individual analytes

	parent ion	CE (eV)	product ions
JAS-2 <sub>met</sub>	319.1	-16.0; -29.0	187.2; 142.15
JAS-2	282.8	-13.0; -32.0; -42.0	169.05; 84.05; 55.0
DEX	269.1	-13.0	155.0

CE = collision energy

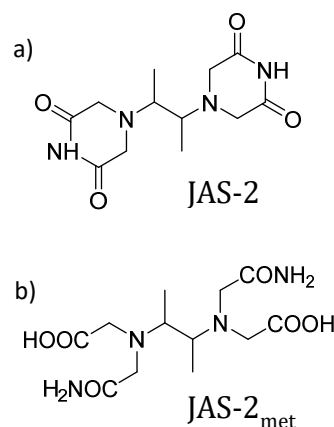
analytical method capable of determination of this compound in biological materials to investigate its fate in cardiomyocytes and an organism. The objective of this study is to develop a pilot UHPLC-MS/MS conditions for determination of JAS-2 and its metabolite (*N,N'*-bis(carbamoylmethyl)-2,3-diaminobutane-*N,N'*-diacetic acid, JAS-2<sub>met</sub>, Fig. 2b) in the solution and cell culture medium (DMEM).

## 2. Experimental

JAS-2 was synthesized via six-step procedure starting from meso-2,3-butanediol. The crucial step of the preparation, cyclization of terminal piperazine-2,6-diones, was performed according to method of R. M. Snapka et al [7]. The two rings opened metabolite (JAS-2<sub>met</sub>) was prepared analogically to ADR-925 by alkaline hydrolysis of JAS-2 and subsequent acidification using the ion exchanger Amberlyst 15. Identity and purity were verified by NMR and MS techniques. The stock solutions were prepared using solvents DMSO, methanol 50% and methanol 100% for JAS-2, JAS-2<sub>met</sub> and DEX respectively.

The analyses were performed using a UHPLC system (Nexera, Shimadzu, Japan) coupled with a triple quadrupole mass spectrometer with ESI ion source (LCMS-8030, Shimadzu, Japan). Following chromatographic columns were tested during the method development: Atlantis HILIC Silica (150 x 2.1 mm, 3 μm, Waters), Cogent 2.0 Bidentate C18 (100 x 2.1 mm, 2.2 μm, MicroSolv Technology Corporation), Luna Omega Polar (100 x 2.1 mm, 1.6 μm, Phenomenex), Luna Omega PS (100 x 3 mm, 5 μm, Phenomenex), Synergi Polar (100 x 3.0 mm, 2.5 μm, Phenomenex), Zorbax SB Aq (3x100

mm, 1.8 μm, Agilent) . The mobile phase was composed of a mixture of either water or 0.5 - 10 mM ammonium formate (A) and methanol or acetonitrile (B) in a different gradient profiles. DEX was utilized as an internal standard for JAS-2. ADR-925 (Fig 1b) and 1,2-diaminopropane-*N,N,N',N'*-tetraacetic acid (PDTA, Fig 2a) were tested as a potential internal standards for JAS-2<sub>met</sub>. The quantification was done using SRM for higher selectivity (Tab 1). Various dilution ratio (5x, 10x, 20x) of cell culture media - DMEM (Sigma Aldrich, Germany) were tested during sample preparation development using different concentrations of



methanol (20%, 50% and 100%).

Figure 2. JAS-2 (a), a novel analogue of the cardioprotective drug dexrazoxane and its metabolite (b).

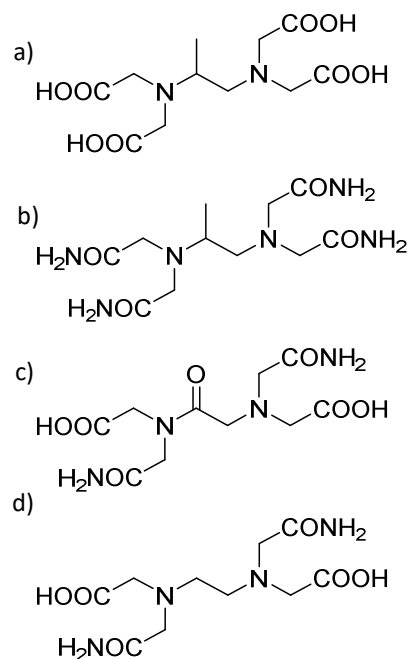


Figure 3. PDTA - one of the tested internal standards (a). Other compounds that will be tested as internal standards – JR281B (b), JR-H2 (c), GK-657 (d).

Linearity of the method was evaluated over the concentration range from 5 to 150  $\mu\text{mol/l}$  and 10 to 125  $\mu\text{mol/l}$  for JAS-2 and JAS-2<sub>met</sub>, respectively, using linear regression for each compound separately and together in a mixture.

### 3. Results and Discussion

The main complication of simultaneous analysis of JAS-2 and its metabolite was distinct polarities of these analytes, which lead to their completely different retention behavior. Moreover, the high polarity of JAS-2<sub>met</sub> and its chelation properties resulted in poor retention of the compound on reversed phase columns and weak peak shapes.

Luna Omega Polar (100 x 2.1 mm, 1.6  $\mu\text{m}$ , Phenomenex) was finally selected as a stationary phase because it provided the best separation properties and peak shapes for such different analytes. DEX was chosen as internal standard for JAS-2. The structurally close analogues of the metabolite (ADR-925 and PDTA) could not be used as internal standards for JAS-2<sub>met</sub> as their retention behavior resulting in JAS-2<sub>met</sub> peak overlapping, associated with signal suppression and poor repeatability of both tested internal standards. Hence the linearity for JAS-2<sub>met</sub> had to be tested without using an internal standard. 1,2-Diaminopropane-*N,N,N',N'*-tetraacetamide (JR281B Fig 3b), *N,N'*-bis(carbamoylmethyl)glycinamide-*N,N'*-diacetic acid (JR-H2, Fig 3c) and *N,N'*-bis(carbamoylmethyl)ethylene diamine-*N,N'*-diacetic acid (GK-657, Fig 3d) will be further tested as potential internal standards for JAS-2<sub>met</sub>.

The composition of mobile phase was optimized and 1 mM ammonium formate (A) and methanol (B) in the following gradient: 0-1.5 min - 5% (B); 1.5-3 min - 5-90% (B); 3-5 min 90% (B); 5.01-8 min 5% (B) (Fig 4) were found to be appropriated. The representative chromatogram of separation is shown in Fig 5.

Samples were prepared by dilution of the DMEM medium five times with methanol 50%. Linearity of

the method was proved over the concentration range from 5 to 150  $\mu\text{mol/l}$  with  $R^2 \geq 0,999$  and from 10 to 125  $\mu\text{mol/l}$  with  $R^2 \geq 0,996$  for JAS-2 and JAS-2<sub>met</sub>, respectively.

### 4. Conclusion

The novel UHPLC-MS/MS conditions for determination of JAS-2 and its metabolite in DMEM cell culture medium was developed. After selection of an internal standard for the metabolite, this condition will be utilized for stability/activation study in DMEM medium. Moreover, this study provides a basis for further optimization and validation of the bioanalytical method for analyses of JAS-2 and its metabolite in different relevant biological materials (e.g. plasma, heart tissue, etc). This study brings novel data to investigation of cardioprotective analogue of DEX, JAS-2 and its application in further research may help to clarify the structure-effect relationship in the group of DEX analogues.

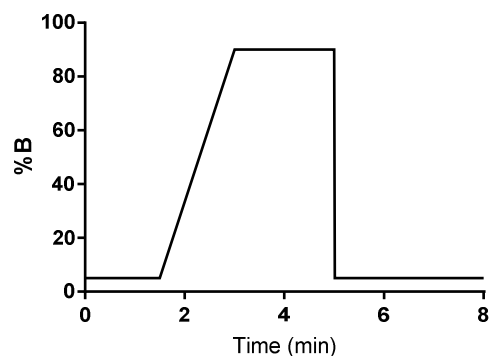


Figure 4. Gradient profile of the mobile phase, expressed as a concentration of the organic solvent (B) as a function of time.

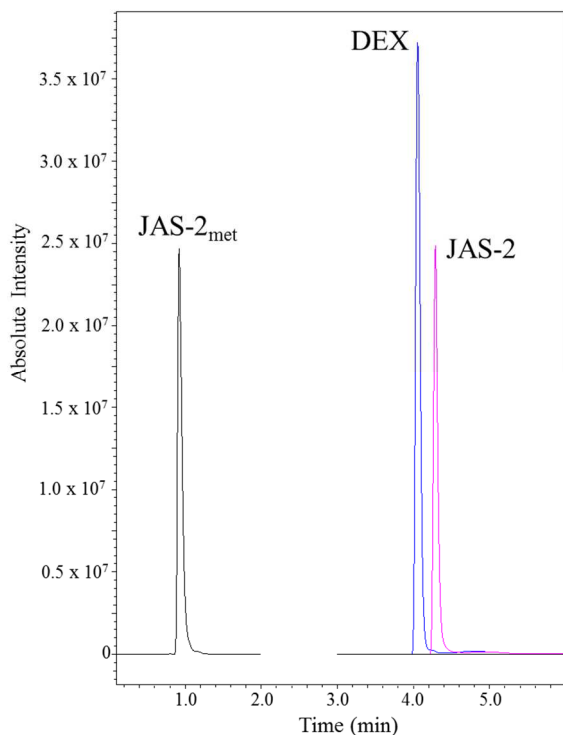


Figure 5. The representative chromatogram of separation.

### Acknowledgement

The research was supported by the Charles University in Prague (projects GAUK 1550217 and SVV 260 401).

### REFERENCES

- [1] Sterba, M.; Popelova, O.; Vavrova, A.; Jirkovsky, E.; Kovarikova, P.; Gersl, V.; Simunek, T., Oxidative stress, redox signaling, and metal chelation in anthracycline cardiotoxicity and pharmacological cardioprotection. *Antioxid Redox Signal* 2013, **18** (8), 899-929, 10.1089/ars.2012.4795
- [2] Elliott, P., Pathogenesis of cardiotoxicity induced by anthracyclines. *Semin Oncol* 2006, **33** (3 Suppl 8), S2-7, 10.1053/j.seminoncol.2006.04.020
- [3] Jirkovská-Vávrová, A.; Roh, J.; Lenčová-Popelová, O.; Jirkovský, E.; Hrušková, K.; Potůčková-Macková, E.; Jansová, H.; Hašková, P.; Martinková, P.; Eisner, T.; Kratochvíl, M.; Šús, J.; Macháček, M.; Vostatková-Tichotová, L.; Geršl, V.; Kalinowski, D. S.; Muller, M. T.; Richardson, D. R.; Vávrová, K.; Štěřba, M.; Šimůnek, T., Synthesis and analysis of novel analogues of dexrazoxane and its open-ring hydrolysis product for protection against anthracycline cardiotoxicity *in vitro* and *in vivo*. *Toxicol. Res.* 2015, **4** (4), 1098-1114, 10.1039/c5tx00048c
- [4] Zhang, S.; Liu, X.; Bawa-Khalfe, T.; Lu, L. S.; Lyu, Y. L.; Liu, L. F.; Yeh, E. T., Identification of the molecular basis of doxorubicin-induced cardiotoxicity. *Nat Med* 2012, **18** (11), 1639-1642, 10.1038/nm.2919
- [5] Tanabe, K.; Ikegami, Y.; Ishida, R.; Andoh, T., Inhibition of topoisomerase II by antitumor agents bis(2,6-dioxopiperazine) derivatives. *Cancer Res* 1991, **51** (18), 4903-4908
- [6] Vavrova, A.; Jansova, H.; Mackova, E.; Machacek, M.; Haskova, P.; Tichotova, L.; Sterba, M.; Simunek, T., Catalytic inhibitors of topoisomerase II differently modulate the toxicity of anthracyclines in cardiac and cancer cells. *PLoS One* 2013, **8** (10), e76676, 10.1371/journal.pone.0076676
- [7] Snapka, R. M.; Woo, S. H.; Blokhin, A. V.; Witiak, D. T., Inhibition of topoisomerase II by ICRF-193, the meso isomer of 2,3-bis(2,6-dioxopiperazin-4-yl)butane - Critical dependence on 2,3-butanediyl linker absolute configuration. *Biochem Pharmacol* 1996, **52** (4), 543-549, 10.1016/0006-2952(96)00305-X

# P18 *IN VITRO* BIOACTIVATION STUDY OF A NEW ANTIPROLIFERATIVE DRUG SOBUXOXANE IN BIOLOGICAL MATRICES

PETRA REIMEROVÁ<sup>1</sup>, ANNA JIRKOVSKÁ<sup>2</sup>, HANA PISKÁČKOVÁ<sup>1</sup>, GALINA KARABANOVICH<sup>3</sup>, JAROSLAV ROH<sup>3</sup>  
AND PETRA ŠTĚRBOVÁ-KOVAŘÍKOVÁ<sup>1</sup>

<sup>1</sup> Department of Pharmaceutical Chemistry and Pharmaceutical Analysis, Faculty of Pharmacy, Charles University, Akademika Heyrovského 1203, Hradec Králové, 500 05, CZECH REPUBLIC

<sup>2</sup> Department of Biochemical Sciences, Faculty of Pharmacy, Charles University, Akademika Heyrovského 1203, Hradec Králové, 500 05, CZECH REPUBLIC

<sup>3</sup> Department of Organic and Bioorganic Chemistry, Faculty of Pharmacy, Charles University, Akademika Heyrovského 1203, Hradec Králové, 500 05, CZECH REPUBLIC

Abstract – Sobuzoxane was synthesized as a prodrug of an antiproliferative bis-dioxopiperazine drug ICRF-154 (analogue of cardioprotectant dexrazoxane) to improve its bioavailability. Although it is a clinically approved drug in Japan, there are only scarce information on its metabolism and no modern method suitable for comprehensive biodegradation study. Therefore, we developed first UHPLC-MS/MS method for analysis of these compounds in relevant biological material and applied it to an *in vitro* biodegradation experiment in cardiac cells and plasma. Because of suspected rapid degradation of the prodrug, sample preparation consisted of simple protein precipitation. The *in vitro* experiment confirmed that sobuzoxane is in biological matrices rapidly metabolized to the active compound and then further to EDTA-like chelating agent.

**Keywords:** bis-dioxopiperazines, sobuzoxane, biodegradation, UHPLC-MS/MS

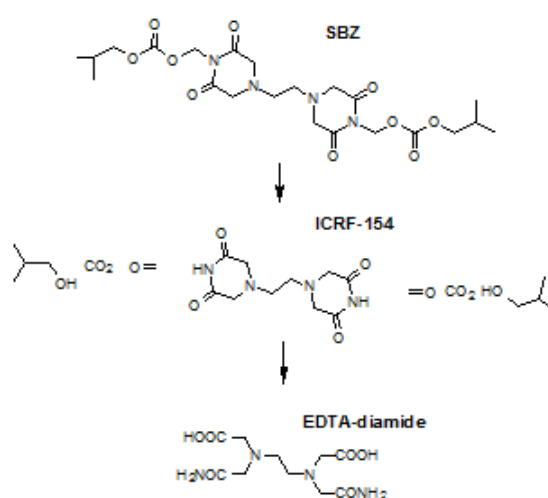
## 1. Introduction

Analogues of bis-dioxopiperazine were developed as potential anticancer drugs – inhibitors of topoisomerase II, an important enzyme for DNA replication that makes it a perfect target for cancer therapy<sup>[1]</sup>. One of bis-dioxopiperazines, namely dexrazoxane, was found to be effective in preventing anthracycline induced cardiotoxicity and was approved for the use in clinical practice as a cardioprotective agent<sup>[2]</sup>. The exact mechanism of cardioprotective action is still unknown. Moreover, the development of bis-dioxopiperazines is significantly complicated by poor bioavailability and solubility.

Sobuzoxane (**SBZ**, 4,4'-(1,2-ethanediyl)bis(1-isobutoxycarbonyloxmethyl-2,6-piperazinedione), *Fig.1*) has been synthesized as a prodrug of 1,1'-ethylendi-3,5-dioxopiperazine (**ICRF-154**, *Fig.1*) to increase its lipophilicity and mainly solubility to enable p.o. administration<sup>[3]</sup>. Although SBZ is a clinically approved drug in Japan<sup>[4]</sup>, the data on its bioactivation in biological materials are only scarce. It is probably immediately after administration converted to ICRF-154, formaldehyde and CO<sub>2</sub> are released during this esterase dependent activation (*Fig. 1*)<sup>[5]</sup>. Furthermore, ICRF-154 is likely metabolized in the body to its analogue with open bis-dioxopiperazine rings (**EDTA-diamide**, *Fig.1*) with chelating properties. This metabolism pathway was only hypothesized and it is based on biodegradation data of its close analogue –

dexrazoxane (DEX). DEX metabolic conversion to the chelator - ADR-925 is shown in *Fig. 2*. So far, there was not published any method capable of simultaneous analysis of SBZ and its metabolites (ICRF-154, EDTA-diamide) in relevant biological materials that could be used for comprehensive bioactivation study.

The aim of our study was to develop and validate UHPLC-MS/MS method for simultaneous analysis of SBZ, ICRF-154 and EDTA-diamide. This method was applied to a pilot bioactivation experiment with SBZ in neonatal ventricular rat cardiomyocytes (NVCM) and plasma and the results were compared with bioactivation of DEX.



*Figure 1* Biodegradation of sobuzoxane (SBZ) and structures of its metabolites – ICRF-154 and EDTA-diamide

Table 1 Selected precursor and product ions of studied compounds and their internal standards used for quantitation

Compound	Mw (g/mol)	SRM
SBZ	514	532.3 → 341.1
ICRF-154	254	255.2 → 141.1
EDTA-diamide	289	290.9 → 158.9
IS-SBZ	486	504.0 → 383.1
DEX	268	269.1 → 155.2
ADR-925	304	305.1 → 173.1

## 2. Experimental

### 2.1. Samples and Measurements

Studied compounds and their internal standards were synthesized at the Department of Organic and Bioorganic Chemistry of Faculty of Pharmacy (Charles University, Czech Republic) and their identity was verified using NMR and MS. The rest of the substances and LC-MS solvents used in this study were purchased from Sigma-Aldrich (Germany).

All samples were analyzed using a Nexera X2 UHPLC system coupled with LCMS-8030 triple quadrupole mass spectrometer with ESI ion source operating in a positive mode (Shimadzu, Japan). The acquired data were processed using LabSolutions software (v. 5.60 SP2, 2013, Shimadzu, Japan). Chromatographic separation was achieved on a Zorbax SB-Aq column (100 x 3 mm, 1.8 μm, Agilent) with the same type of a guard column. The column was flushed with a mixture of 2 mM EDTA solution and acetonitrile (90:10, v/v) before its first use to remove ions that may interfere with analysis of analyte with chelating properties (EDTA-diamide). Mobile phase composed of (A) 1 mM ammonium formate and (B) methanol was employed in a following gradient: 0 - 1.5 min (5% B), 1.5 - 5 min (5-90% B), 8.51 - 13 min (5% B). A flow rate of 0.3 ml/min, a column thermostat temperature of 30°C and an injection volume of 3 μl were used. Autosampler temperature was set to 15°C. Quantitation was performed in selected reaction monitoring mode (SRM) using low resolution (see Table 1). Close structural analogues of studied compounds were utilized as internal standards – ADR-925, dexrazoxane and 4,4'-(1,2-ethanediyl)bis(N1-propoxycarbonyloxmethyl-2,6-

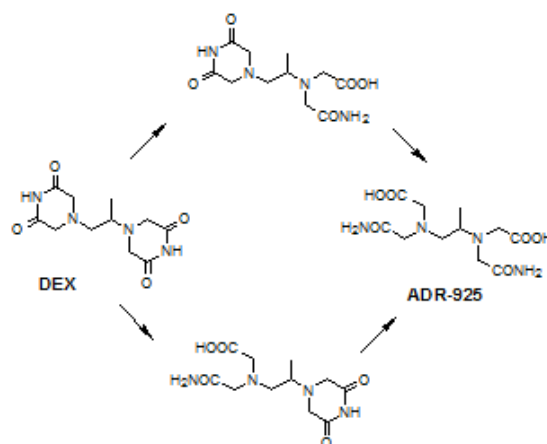


Figure 2 Biodegradation of dexrazoxane (DEX) and structure of its metabolite ADR-925

piperazinedione) for EDTA-diamide, ICRF-154 and sobuzoxane, respectively (Figs. 2 and 3).

Biodegradation study of sobuzoxane was conducted in following biological matrices and concentrations of the drug: neonatal ventricular rat cardiomyocytes – NVCM (100 μM), DMEM cell culture medium (30 μM, 100 μM) and rabbit plasma (100 μM). Experiment was followed by stability study of ICRF-154 (50 μM) in DMEM cell culture medium and rabbit plasma. ADS buffer (pH 7.4) and PBS buffer (pH 7.4) were used as a control media in this study for DMEM medium and rabbit plasma, respectively. All experiments were conducted at 37°C, NVCM cells were incubated in 5% CO<sub>2</sub> (CelCulture® Incubator – ESCO, USA).

Biological matrices used in biodegradation study were treated by protein precipitation or simple dilution. Because of low post-preparative stability of sobuzoxane, 0.1% formic acid was added to both precipitation and dilution media. As formic acid was incompatible with analysis of EDTA-diamide, this metabolite was analyzed separately from ICRF-154 and sobuzoxane. The NVCM cells were sonicated for 3 min. Then 300 μl of pure ice-cold

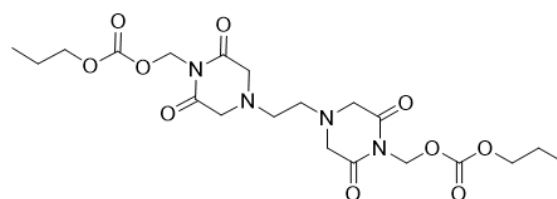


Figure 3 Structure of internal standard for sobuzoxane (IS-sobuzoxane)

methanol was added and the precipitate was centrifuged for 10 min (10,000 rpm, 4°C). Supernatant was filtered (0.22 µm), divided into two parts and 0.1% of formic acid was added for the analysis of SBZ and ICRF-154. DMEM cell culture medium and buffers (PBS, ADS) were diluted 20x with 20% methanol with or without addition of 0.1% of formic acid and injected. Plasma samples were precipitated with 150 µl of ice-cold methanol and centrifuged for 10 min (10,000 rpm, 4°C). Supernatant was filtered (0.22 µm) and injected. The method was validated within concentration ranges relevant for the bioactivation study by testing linearity, precision and accuracy according to FDA guideline on Bioanalytical Method Validation [6] and fitted well the criteria.

### 3. Results and Discussion

#### 3.1. Experiments

UHPLC-MS/MS method development was hindered mainly by distinct polarity of the analytes and chelation properties of EDTA-diamide that resulted in its poor sensitivity. Gradient mode was utilized for optimal separation (Fig.4) and the chromatographic column was flushed with another chelator - EDTA prior to first use to decrease the amount of metals present in the system. Furthermore, formic acid used as a solvent for ICRF-154, because of its low solubility, suppressed ionization of EDTA-diamide. Therefore, for the verification of the optimal chromatographic conditions as well as stability and biodegradation study saturated solution of ICRF-154 in water was used. Because of its low concentration, stability of ICRF-154 in DMEM and rabbit plasma was tested at 50 µM concentration to avoid excessive dilution of the matrix. For comparison with sobuzoxane and dexrazoxane the results were expressed in % of initial concentration.

Stability of SBZ (100 µM, 30 µM) in DMEM cell culture medium and ADS buffer (as a control) was examined. Equally slow degradation of SBZ was observed in both media and the decrease of concentration of SBZ was comparable at both tested concentrations. No ICRF-154 was detected after 24 hours of incubation. On the other hand low amount of EDTA-diamide was found at the last time

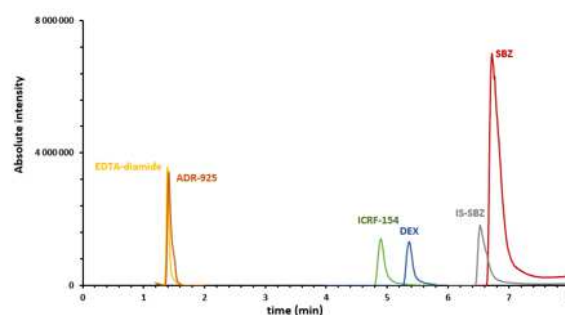


Figure 4 Example of chromatographic separation of sobuzoxane, ICRF-154, EDTA-diamide and their internal standards

interval but it was not equimolar to the fall in SBZ concentration. This may be explained by poor solubility of ICRF-154. Stability experiment of ICRF-154 (50 µM) in DMEM medium showed gradual equimolar conversion of the drug to its open-ringed analogue – EDTA-diamide. This confirmed our hypothesis of similar degradation behavior of ICRF-154 compared to dexrazoxane.

Next, biodegradation of SBZ in cardiac cells was studied using incubation of the drug (100 µM) with NVCM in DMEM cell culture medium. At selected time intervals DMEM medium was sampled and diluted before analysis (see Experimental). The NVCM cells were harvested and flushed with ice-cold PBS before sample treatment. Low concentration of sobuzoxane was detected in NVCM cells at the beginning of the experiment and after 6-hour incubation period the SBZ concentration decreased below the detection limit of the UHPLC-MS/MS method. Surprisingly high concentrations of ICRF-154 were found in the cells even at the first time interval which suggests rapid biodegradation of SBZ by the cells. ICRF-154 was then gradually metabolized to EDTA-diamide. Concentration of SBZ in DMEM medium in which the NVCM cells were incubated decreased more quickly compared to the DMEM medium without the cells. Also, low concentrations of ICRF-154 were detected and slightly higher concentration of EDTA-diamide was found in this medium. Comparison of this experiment with our previous data on incubation of DEX (100 µM) with NVCM cells showed that by using a prodrug (sobuzoxane), higher concentrations of the active compound (ICRF-154) can be achieved in the cardiac cells. It is probably caused by higher lipophilicity of the prodrug which enables better passage through the cell membrane.

*In vitro* experiment in rabbit plasma confirmed that SBZ is quickly metabolized to ICRF-154 as the prodrug was not detectable even after 8 minutes of incubation and appropriate gain of ICRF-154 was found. It was then relatively slowly converted to EDTA-diamide. On the other hand, concentration of SBZ in PBS decreased slowly, no ICRF-154 was detected in the samples and only low amount of EDTA-diamide was found after 24 hours. The slower biodegradation of ICRF-154 was verified by an *in vitro* experiment in rabbit plasma when 50% of the initial concentration (50 µM) was detected after 6 hours of incubation at 37°C.

The stability of sobuzoxane in DMEM medium without the cells, ADS and PBS buffers which were all used as a controls for the biodegradation experiments was comparable. After 24 hours the concentration of sobuzoxane relatively slowly decreased under its LLOQ, but no ICRF-154 and only small amount of EDTA-diamide were detected.

#### 4. Conclusion

Novel UHPLC-MS/MS method for simultaneous analysis of sobuzoxane and its potential metabolites (ICRF-154 and EDTA-diamide) was developed and validated. It was then utilized for a pilot biodegradation study in neonatal ventricular rat cardiomyocytes and rabbit plasma (cell culture medium, PBS and ADS buffers were used as a control). It was found that the prodrug sobuzoxane is rapidly metabolized to its active compound in both matrices. ICRF-154 is then relatively slowly converted into EDTA-like chelating compound – EDTA-diamide. This biodegradation process is similar to that of its bis-dioxopiperazine analogue dexrazoxane. This study provides a basic tool for investigation of the fate of the sobuzoane in relevant biological material and may help shed more light into the drug bioactivation *in vitro*. These results will be further utilized in *in vivo* study of

metabolism of sobuzoxane in rabbits. They may also advance the development of new cardioprotective drugs and help understanding the full mechanism of action of bis-dioxopiperazines.

#### Acknowledgement

This research was supported by Charles University (projects GAUK 344 615 and SVV 260 401).

#### REFERENCES

- [8] Larsen, A.K.; Escargueil, A.E.; Skladanowski, A.: Catalytic topoisomerase II inhibitors in cancer therapy. *Pharmacol Ther.* 2003 99(2), 167-81 DOI 10.1016/S0163-7258(03)00058-5
- [9] Testore, F.; Milanese, S.; Ceste, M.; de Conciliis, E.; Parello, P.; Lanfranco, C.; Manfredi, M.; Ferrero, G.; Simoni, C.; Miglietta, L.; Ferro, S.; Giarretto, L.; Bosso, G.: Cardioprotective effect of dexrazoxane in patients with breast cancer treated with anthracyclines in adjuvant setting: a 10-year single institution experience. *Am J Cardiovasc Drugs.* 2008 8(4), 257-63 DOI 10.2165/00129784-200808040-00005
- [10] Narita, T.; Koide, Y.; Yaguchi, S.; Kimura, S.; Izumisawa, Y.; Takase, M.; Inaba, M.; Tsukagoshi, S.: Antitumor activities and schedule dependence of orally administered MST-16, a novel derivative of bis(2,6-dioxopiperazine). *Cancer Chemother Pharmacol.* 1991 28(4), 235-40
- [11] Ohno, R.; Masaoka, T.; Shirakawa, S.; Sakamoto, S.; Hirano, M.; Hanada, S.; Yasunaga, K.; Yokomaku, S.; Mitomo, Y.; Nagai, K.; Yamada, K.; Furue, H.: Treatment of adult T-cell leukemia/lymphoma with MST-16, a new oral antitumor drug and a derivative of bis(2,6-dioxopiperazine). The MST-16 study group. *Cancer* 1993 71(7), 2217-21 DOI 10.1002/1097-0142(19930401)71:7<2217::AID-CNCR2820710709>3.0.CO;2-8
- [12] Swift, L.P.; Cutts, S.M.; Nudelman, A.; Levovich, I.; Rephaeli, A.; Phillips, D.R.: The cardio-protecting agent and topoisomerase II catalytic inhibitor sobuzoxane enhances doxorubicin-DNA adduct mediated cytotoxicity. *Cancer Chemother Pharmacol.* 2008 61(5), 739-49 DOI 10.1007/s00280-007-0528-2
- [13] <https://www.fda.gov/downloads/Drugs/Guidance/ucm070107.pdf> (cited 28.9.2017)



# P19 COMBINATION OF PREPARATIVE ISOELECTRIC FOCUSING AND MALDI-TOF MS FOR IDENTIFICATION OF COLORED BACTERIA

JIŘÍ ŠALPLACHTA,<sup>1\*</sup> MARIE HORKÁ,<sup>1</sup> FILIP RŮŽIČKA,<sup>2</sup> AND KAREL ŠLAIS<sup>1</sup>

1 Department of fluid phase separations, Institute of Analytical Chemistry of the CAS, v. v. i., Veveří 97, Brno, 60200, Czech Republic

2 Department of microbiology, Faculty of Medicine, Masaryk University, Kamenice 5, Brno, 62500, Czech Republic

**Abstract** – This study describes a preparative method based on isoelectric focusing (IEF) of colored microorganisms in a cellulose-based separation medium. Three opportunistic human pathogens, *Micrococcus luteus*, *Dietzia* sp. and *Rhodotorula mucilaginosa*, were first analyzed by preparative IEF. Zones of the separation medium with microbial cells were collected with a spatula, simply processed and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

**Keywords:** bacteria, Isoelectric focusing, mass spectrometry

## 1. Introduction

Identification of microorganisms from a complicated matrix is required for diagnostic purposes in medicine, environmental studies, food industry, and many other areas. Many different conventional techniques, such as polymerase chain reaction, DNA-typing, specific antibodies, are used for characterization of variety of microorganisms [1,2]. In recent years, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has become a fast and reliable technique for identification of various microorganisms including pathogenic ones [3]. Nevertheless, sample preparation, including pre-separation and pre-concentration techniques, is still a crucial step in the analysis of many microbial samples.

Electrophoretic techniques can be advantageously used for both the pre-concentration and separation of microorganisms [4]. Isoelectric focusing (IEF) is an example of technique suitable for separation and pre-concentration of microorganisms. The IEF separates microbial cells according to their different isoelectric points (pI) and it also increases concentration of the separated microorganisms during focusing [4,5]. The microorganisms are usually separated in the capillary format of IEF (CIEF) [5]. However, the use of this technique is somewhat limited by the small volume of the sample which is injected into the capillary.

The objective of this study is to demonstrate the ability of method combining recently developed preparative IEF in a cellulose-based separation medium [6] with a MALDI-TOF MS analysis to identify microorganisms. Three colored

microorganisms, two bacteria (*Micrococcus luteus* CCM 169T and *Dietzia* sp. CCM 2585) and one yeast (*Rhodotorula mucilaginosa* M019), were selected for this purpose. These microorganisms are opportunistic human pathogens found especially in immunocompromised patients [7-9].

## 2. Experimental

### 2.1. Chemicals

The vast majority of chemicals, including all the chemicals for preparative IEF, were purchased from Sigma-Aldrich (Schnelldorf, Germany). 3,5-Dimethoxy-4-hydroxycinnamic acid (SA) and protein calibration mixture ProMix2 were purchased from LaserBio Labs (Sophia-Antipolis Cedex, France). Colored pI markers for preparative IEF pI 2.0 (green), 6.2 (red), 10.1 (violet) were developed and synthesized at the Institute of Analytical Chemistry of the CAS, v. v. i. All chemicals were of analytical or MS grade.

### 2.2. Bacterial strains and growth conditions

The strains of *Micrococcus luteus* CCM 169T, and *Dietzia* sp. CCM 2585 were obtained from the Czech Collection of Microorganisms (Brno, Czech Republic). The colored strain *R. mucilaginosa* M019 was isolated from clinical material and stored in a Collection of Microbiology, Masaryk University and St. Anne's University Hospital (Brno, Czech Republic). The tested strains included in this study were stored at  $-70^{\circ}\text{C}$  in Itest cryotubes (ITEST plus, Czech Republic). Before each experiment, the strains were thawed quickly at 37

°C and cultivated on blood or Mueller-Hinton agar (Oxoid, United Kingdom) at 37 °C for 72 hours.

### 2.3. Preparative IEF device and procedure

The preparative IEF device was described previously [6]. Eight hundred microliters of the separation medium together with 30 µL of the *pI* marker solution (*pI* 2.0, 6.2 and 10.1, each of them 5 µg mL<sup>-1</sup>) were introduced in an empty V-shaped plastic trough was positioned on a power source and fixed by inserting the working electrodes into the trough. The power source was switched on at time *t*<sub>0</sub>. The trough was covered with a plastic lid to retard the evaporation of water from the separation medium. After two hours, the plastic lid was removed and 100 µL of the microbial sample was introduced along the central third of the trough at time *t*<sub>inj</sub> = *t*<sub>0</sub> + 2 h. The sample was either individual microorganism or a mixture of the microorganisms resuspended in a physiological saline solution (PSS). The IEF device was left running for additional 18 h and then the fractions, defined by the positions of colored *pI* markers, were collected and analyzed by MALDI-TOF MS.

### 2.4. Fraction processing for MALDI-TOF MS

The colored part of the bed with the separated cells was harvested by a spatula and inserted into the eppendorf tube. The fraction was overlaid with 100 µL of distilled water and centrifuged at 1000×g for 5 min on the MiniSpinPlus (Eppendorf), then at 3000×g for 10 min, and subsequently at 6000×g for 20 min. The pellet was used for MALDI-TOF MS analysis.

### 2.5. MALDI-TOF MS

Cultivated microbial strains were resuspended in deionized water to the final concentration of 1 × 10<sup>8</sup> cells mL<sup>-1</sup>. Fifty microliters of the bacterial suspension was centrifuged at 6000×g for 20 min, the supernatant was discarded and the pellet was resuspended in 50 µL of SA solution (20 mg mL<sup>-1</sup> in ACN/0.1% TFA, 3:2, (v/v)). The colored part of the bed with the separated cells was harvested by a spatula and inserted into the eppendorf tube. The fraction was overlaid with 100 µL of distilled water and centrifuged at 6000×g for 20 min. The supernatant was discarded and the cells were

resuspended in 10 µL of SA solution. After a brief shaking, all microbial samples were centrifuged again at 3000×g for 3 min and the supernatants (0.7 µL each) were spotted onto a sample plate previously overlaid with the SA solution and left to dry at room temperature. All MS experiments were performed on the AB Sciex TOF/TOF 5800 System operating in a linear positive ion mode.

## 3. Results and Discussion

### 3.1. Preparative IEF

Preparative IEF of a blank sample of PSS, yellow cells of *M. luteus*, cinnamic cells of *R. mucilaginosa* and pink cells of *Dietzia* sp., each of them re-suspended in PSS, are shown in the photo of the trough in Figs. 1A-D. PSS as a blank sample and microbial samples were injected into the trough at time *t*<sub>0</sub>+2h. Focused zones of *pI* markers can be observed at time *t*<sub>0</sub>+20h. The position of cell zones was localized directly visually or by a comparison of the zones of known *pI* values of *pI* markers and the cells. The yellow zone of the focused cells of *M. luteus* was found in the acidic part of the cellulose bed corresponding to a *pI* lower than 2.0, green zone of *pI* marker. Similarly, the focusing process can be observed in Figs. 2C and 2D. Thanks to a greater color contrast of white cellulose vs. cinnamic cells of *R. mucilaginosa*, the zone of the cinnamic cells becomes visible already at time *t*<sub>0</sub>+3h (Fig. 2C). Fully focused zones, cinnamic and pink, are visible after twenty hours, see Figs. 2C or 2D, respectively.

The separation bed has a gel-like appearance after the focusing run so the fractions containing microbial cells can be easily collected by a spatula. The cells from the fractions were further analyzed by MALDI-TOF MS.

### 3.2. MALDI-TOF MS

The cultivated microbes taken from the Petri dish were first analyzed by MALDI-TOF MS in order to obtain reference mass spectra of the individual microorganisms (Figs. 2A, 3A and 4A). Then the single strains were analyzed by preparative IEF, fractions were collected and analyzed by MALDI-TOF MS. The microbial cells were easily extracted from the collected fractions with water further processed for MALDI analysis. Figs. 2B, 3B and 4B show the mass spectra of *R. mucilaginosa*, *M.*

*luteus*, and *Dietzia* sp., respectively, after their analysis by preparative IEF. With respect to *R. mucilaginosa* and *M. luteus* (Figs. 2B and 3B, respectively) most of the mass signals detected in the mass spectra of the individual strains were also detected in the mass spectra of the same strains after their analysis by preparative IEF. Unfortunately, low-quality mass spectra were obtained in the case of *Dietzia* sp. (Fig. 4B). Nevertheless, major peaks were detected in the mass spectra of *Dietzia* sp. analyzed both before and after preparative IEF. It also follows from the obtained data of all the examined strains that some of the shared mass signals differ in their intensities and, moreover, there are some mass signals detected only in one type of the sample, in the mass spectra of either microbial strains or IEF fractions. It seems that some cells are changed during preparative IEF (e.g., cell disruption can occur) which results in changes in the mass profile.

Then, all three strains together were separated by preparative IEF, respective fractions were collected and analyzed by MALDI-TOF MS. Obtained mass spectra of the fractions of *R. mucilaginosa*, *M. luteus*, and *Dietzia* sp. are shown in Figs. 2C, 3C and 4C, respectively. These mass patterns correspond to those obtained by the MS analysis of the fractions collected after preparative IEF of single strains (Figs. 2B, 3B and 4B). Analogous to the previous analyses described above, there are some differences, rather in the intensities of the mass signals than in the detected mass signals themselves, in the mass spectra of IEF fractions of the same strains (Figs. 2B vs. 2C, 3B vs. 3C and 4B vs. 4C). Although more obvious differences in both mass signals and their intensities were found in the case of *R. mucilaginosa*, the majority of the detected mass signals are shared by both IEF fractions as can be seen in Figs. 2Ab and 2Ac.

#### 4. Conclusion

The colored microorganisms, *R. mucilaginosa*, *M. luteus*, *Dietzia* sp., both individually and in the mixture, were successfully separated by preparative IEF in cellulose-based separation medium according to their *pI* values. This is made possible by the pores of the separation medium which are large enough for the analytes of  $\mu\text{m}$ 's dimensions to

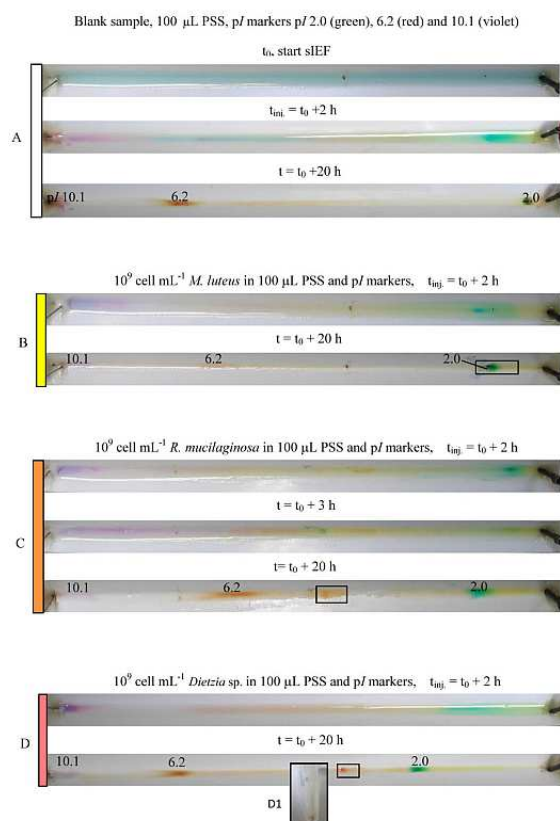


Figure 1. Preparative IEF of the blank sample of PSS (A) *M. luteus* (B), *R. mucilaginosa* (C) and *Dietzia* sp. (D). Sample: 100  $\mu\text{L}$  of PSS (A) or the microbial suspension in PSS (B-D), each of them  $10^9$  cell  $\text{mL}^{-1}$ ; *pI* marker *pI* 2.0, 6.2 and 10.1; (D1) sample prepared from the collected fraction; time:  $t_0$ , the power source switched on;  $t_{inj.} = t_0 + 2$  h, the sample injection time;  $t = t_0 + 3$  or 20 h, the time of focusing run; empty rectangle, collected fraction of the focused zone from the trough.

move in independently of their size. The focused microbial zones in the cellulose bed were collected according to the color of the zones or their isoelectric points. The cells from the zones were further analyzed by MALDI-TOF MS in order to verify the focusing ability of the preparative IEF.

#### Acknowledgement

This work was supported by the Grant Agency of the Czech Republic (Grant No. 16-03749S), Ministry of Health of the Czech Republic (Grant No. 16-29916A), Ministry of the Interior of the Czech Republic (Grant VI20172020069) and by the Czech Academy of Sciences (Institutional Support RVO:68081715).

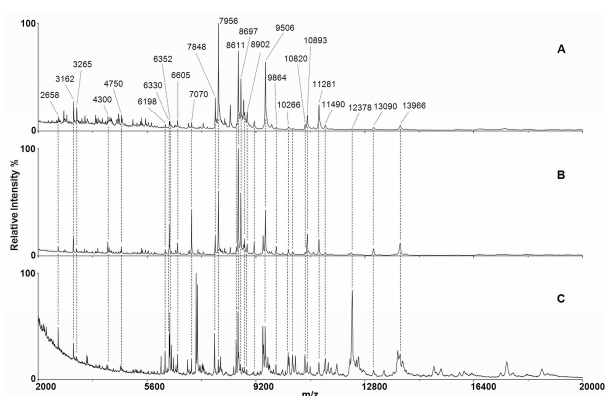


Figure 2. MALDI-TOF mass spectra of *R. mucilaginosa*. (A) cultivated bacterial cells; (B) fraction collected after preparative IEF of single strain; (C) fraction collected after preparative IEF of mixture of three bacterial strains.

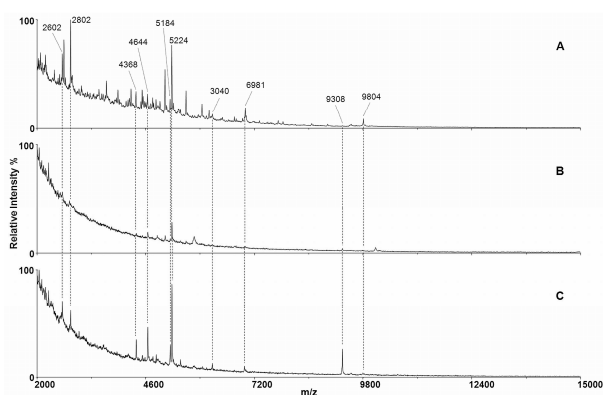


Figure 4. MALDI-TOF mass spectra of *Dietzia* sp. (A) cultivated bacterial cells; (B) fraction collected after preparative IEF of single strain; (C) fraction collected after preparative IEF of mixture of three bacterial strains.

## REFERENCES

- [1] Brondz, I.; Olsen, I.: Microbial chemotaxonomy - chromatography, electrophoresis and relevant profiling techniques, *J. Chromatogr.*, 1986 **379**, 367–411
- [2] Miljković-Selimović, B.; Kocić, B.; Babić, T.; Ristić, L.: Bacterial typing methods, *Fac. Med. Naissensis*, 2009 **26**, 225–233

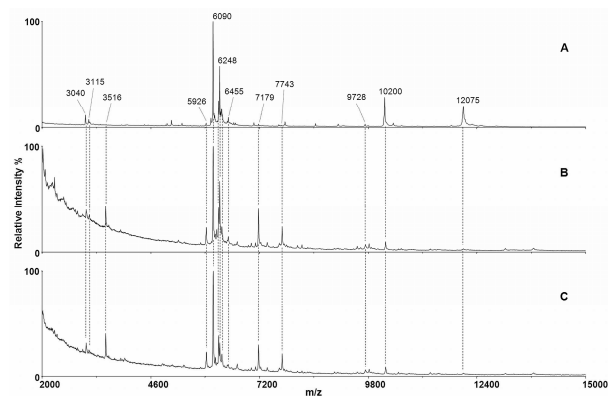


Figure 3. MALDI-TOF mass spectra of *M. luteus*. (A) cultivated bacterial cells; (B) fraction collected after preparative IEF of single strain; (C) fraction collected after preparative IEF of mixture of three bacterial strains.

- [3] Wieser, A.; Schubert, S.: MALDI-TOF MS entering the microbiological diagnostic laboratory –from fast identification to resistance testing, *Trends Anal. Chem.*, 2016 **84**, 80–87
- [4] Szumski, A.; Klodzinska, E.; Buszewski, B.: Separation of microorganisms using electromigration techniques, *J. Chromatogr. A*, 2005 **1084**, 186–193
- [5] Šalplachta, J.; Kubesová, A.; Horká, M.: Latest improvements in CIEF: From proteins to microorganisms, *Proteomics*, 2012 **12**, 2927–2936
- [6] Šalplachta, J.; Horká, M.; Šlais, K.: Preparative isoelectric focusing in a cellulose-based separation medium, *J. Sep. Sci.*, 2017 **40**, 2498–2505
- [7] Greenblatt, C.L.; Baum, J.; Klein, B.Y.; Nachshon, S.; Koltunov, V.; Cano, R.J.: *Micrococcus luteus* – Survival in Amber, *Microb. Ecol.*, 2004 **48**, 120–127
- [8] Duckworth, A.W.; Grant, S.; Grant, W.D.; Jones, B.E.; Meier, D.: *Dietzia natronolimnaios* sp. nov., a new member of the genus *Dietzia* isolated from an East African soda lake, *Extremophiles*, 1998 **2**, 359–366
- [9] Fell, J.W.; Boekhout, T.; Fonseca, A.; Scorzetti, G.; Statzell-Tallman, A.: Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis, *Int. J. Syst. Evol. Microbiol.*, 2000 **50**, 1351–1371

# P20 DETECTING MONO-PHOSPHORYLATED ISOBARIC PEPTIDES USING CESI-MS

BETTINA SARG, KLAUS FASERL, AND HERBERT H. LINDNER\*

Division of Clinical Biochemistry, Medical University of Innsbruck, Innsbruck, A-6020, AUSTRIA

**Abstract** – Here we applied a low-flow capillary electrophoresis (CE) –electrospray ionization interface coupled to a mass spectrometer to analyze mono-phosphorylated isobaric peptides, still a very challenging task in the proteomics field, as side specific product ions are often weak or not even detectable. If such positional isomers cannot be separated, an unambiguous assignment of site-specific phosphorylation effects is problematic, a quantification often impossible. In the course of a kinase activity study isobaric mono-phosphorylated peptides were identified as potential substrates and therefore, the CE-MS approach was evaluated for their identification and quantification.

**Keywords:** capillary electrophoresis-mass spectrometry, post-translational modifications, phosphorylation, isobaric peptides, quantification

## 1. Introduction

It is known that CZE is able to separate peptides with identical amino acid composition but with different sequence provided the sequence variation causes a variation in pKa values of the corresponding peptides. This can be induced, e.g. by local differences in hydrophobicity, conformation, extent of hydration and even more importantly by interaction between charged moieties. For this reason, variation of buffer pH and concentration and the addition of organic solvents, e.g. TFE can be used to optimize resolution. Moreover, the number and position of adjacent charges present either at the amino terminus or in the interior of the peptide may selectively alter the overall charge of those peptides. In this study, CE-MS was used as complementary approach for the quantification of mono-phosphorylated isobaric peptides, still a very challenging task in the proteomics field.

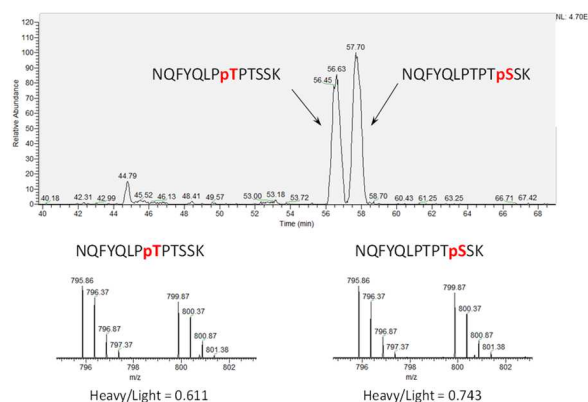
## 2. Experimental

A CESI 8000 (Sciex, Brea, CA) equipped with a neutrally-coated fused-silica capillary with a porous tip acting as nanospray emitter (Sciex, Brea, CA) (total length: 90 cm, i.d.: 30  $\mu$ m, o.d.: 150  $\mu$ m) was coupled via an ESI module to a Thermo Scientific LTQ Orbitrap XL ETD (Bremen, Germany). Prior to each analysis the system was rinsed with background electrolyte (10% acetic acid) for 3 min at 50 psi. The sample was introduced by applying a pressure of 5 psi for 50 sec (40 nL injection volume) followed by a plug of BGE (5 psi for 5 sec). Capillary electrophoresis was conducted by

applying +30 kV with a simultaneous pressure of 1 psi for 60 min at the capillary inlet. The flow rate was determined to be approximately 10 nL/min.

## 3. Results and Analysis

Metabolically labeled peptides obtained after Lys-C cleavage from two yeast strains were pre-fractionated by RP-HPLC and the fractions of interest were analyzed by CE-MS using a neutral capillary [1]. Among other interesting candidates as potential kinase substrates, six phosphopeptide isomers were identified that differ in their migration time by at least 0.4 min. Figure 1 shows the analysis of peptide NQFYQLPPTSSK, which was found to be either phosphorylated at Thr-8 or Ser-11. The individual isobaric phosphopeptides were mutually clearly separated. The lower electrophoretic mobility was consistently found for the isomer with the phosphate group bound to a serine or threonine residue being in close vicinity to a basic residue or the N-terminus.



*Figure 1.* CE-MS separation of phosphopeptide isomers. Extracted ion electropherogram of the peptide: NQFYQLPPTSSK mono-phosphorylated on two different sites; quantification spectra and H/L ratios of the two SILAC labeled phosphopeptide pairs.

\*Correspondence: [Herbert.Lindner@i-med.ac.at](mailto:Herbert.Lindner@i-med.ac.at)

As a further example, the CE-MS analysis of a peptide (SPTLASTDDINSASASVNSHATSVK), which showed even three different phosphorylation sites is depicted in Figure 2. The peptide phosphorylated on Ser-6 shows the highest mobility, followed by the serine peptide modified at Ser-23 close to the C-terminal Lys-25. The peptide with the phosphorylated N-terminal exhibits the lowest electrophoretic mobility. It should be noted that also other structural effects could selectively influence the migration behavior; however, further and more extended studies are necessary to verify such relationships. Based on the ability of CZE to separate the three positional isomers an unambiguous identification of the corresponding phosphorylation site along with a reliable quantification of all three mono-phosphorylated

peptides was achieved (ratios ranging from H/L = 0.562 to 1.330). The isobaric phosphopeptides were clearly separated from each other and in every case the isomer with a phosphate group bound to serine or threonine residues that were in vicinity to a basic amino acid or the N-terminus exhibited a lower electrophoretic mobility.

#### 4. Conclusion

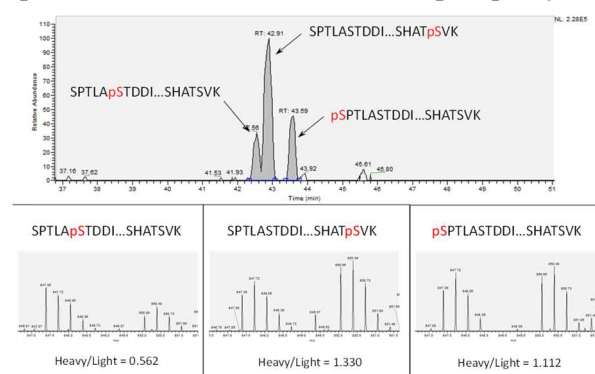
Capillary electrophoresis in combination with accurate MS detection successfully separates positional isomers of mono-phosphorylated peptides allowing unambiguous identification and quantification of individual protein modification sites.

#### Acknowledgement

The authors thank SCIEX Separations for their support.

#### REFERENCES

- [1] Faserl K., Sarg B., Maurer V., Lindner H.H.: Exploiting charge differences for the analysis of challenging post-translational modifications by capillary electrophoresis-mass spectrometry. *J. Chromatogr. A.* 2017 **1498** 215-223 DOI: 10.1016/j.chroma.2017.01.086..



**Figure 2.** CE-MS separation of phosphopeptide isomers. (A) Extracted ion electropherogram of the peptide: SPTLASTDDINSASASVNSHATSVK mono-phosphorylated on three different sites. (B) Quantification spectra and H/L ratios of the three SILAC labeled phosphopeptide pairs.

# P21 COLD PLASMA: THE WAY TO IMPROVE THE SALD ICP MS ANALYSIS REPEATABILITY

MAREK STIBOREK,<sup>1,2</sup> JAN PREISLER,<sup>1,2\*</sup> MASOUD SHEKARGOFTAR,<sup>3</sup> VIKTOR KANICKÝ<sup>1,2</sup> AND JAKUB KELAR<sup>3</sup>

1 Department of Chemistry, Masaryk University, Kamenice 5, Brno, 625 00, Czech Republic

2 CEITEC-Central European Institute of Technology, Kamenice 5, Brno, 625 00, Czech Republic

3 Regional R&D Center for Low-Cost Plasma and Nanotechnology Surface Modifications, Department of Physical Electronics, Masaryk University, Kotlářská 2, Brno, 611 37, Czech Republic

**Abstract** – This work deals with the preparation of model biological microsamples for Substrate Assisted Laser Desorption Inductively Coupled Plasma Mass Spectrometry (SALD ICP MS). This technique provides direct and fast analysis of liquid samples deposited onto polyethylene terephthalate (PET) plate (substrate) in the form of dried droplets with minimal sample preparation and submicrolitre sample volume requirements. Furthermore, SALD allows direct analysis of samples in organic solvents, which cannot be directly nebulised to ICP MS. These benefits are, however, balanced out by low repeatability of the assay, typically about 30 %. One of the approaches to increasing the repeatability is a modification of the substrate surface by Diffuse Coplanar Surface Barrier Discharge (DCSBD) with an addition of an internal standard to the sample. Using this approach, a relative standard deviation under 10 % for model biological samples can be achieved. The only limitation of this technique is the occurrence of the so-called “aging effect”, i.e., the gradual return of the modified physicochemical surface properties to the original state.

**Keywords:** SALD ICP MS, Plasma Treatment, DCSBD, Microsample, Internal Standard

## 1. Introduction

The determination of elements in biological samples is often complicated by complex matrices, presence of organic solvents, and trace amounts of the elements of interest. For this reason, it is necessary to select a technique that bridges the obstacles mentioned above. Such a technique is, for example, Substrate Assisted Laser Desorption Inductively Coupled Plasma Mass Spectrometry (SALD ICP MS), which is capable of analysing liquid samples of submicrolitre volumes in the form of dried droplets (stains) onto a polyethylene terephthalate (PET) substrate [1-3]. The greatest difficulty of this technique is the low repeatability of the assay. This is caused mainly by the poorly reproducible deposition of submicrolitre volumes droplets and the non-homogeneity of the stains produced in terms of the area and the thickness of the solid residue layer in the stain.

The assay repeatability can be increased by increasing the surface energy of the substrate and using internal standards that can correct the mentioned effects.

One way to increase the surface energy, i.e., wettability of the substrate is to expose it briefly to cold

plasma, specifically Diffuse Coplanar Surface Barrier Discharge (DCSBD) [4-5].

The plasma system of the so called DCSBD is composed of regularly alternating silver electrodes on which high alternate voltages are applied. The electrodes are located in a dielectric structure consisting of dielectric oil and thin ceramic plates. The most common arrangement of electrodes in this device is in the form of two intermeshing combs. Surface discharges are then generated on the surface of the ceramic plate, serving as a dielectric barrier. This electrode arrangement (plasma in contact only with inert and highly resistant ceramics) can consist of a theoretically infinite amount of 20x8 cm DCSBD units and thus, it is theoretically possible to reach infinite plasma area.

The principle of substrate surface treatment is based on the excitement and ionization of the species by a high voltage discharge and subsequent introduction of the resulting species onto the substrate surface in the form of radicals, ions and excited molecules.

A disadvantage of this technique over other physicochemical modifications is the gradual decrease of the surface tension compared to the original value prior to the modification, or the so called “aging effect” [6]; this period is in the order of several months. Factors influencing the quality of the modification are the voltage and frequency of the electromagnetic field, the composition of the gaseous atmosphere, the exposure time and the geometry of the sample and electrode.

---

\*Correspondence: preisler@chemi.muni.cz

## 2. Experimental

In the first experiment, a DCSBD device (RPS400-Roplass plasma system 400 W, Roplass, Czech Republic) was used to increase the surface energy of the PET substrate (Bayer MaterialScience, Germany). The substrate surface was modified at 300 W for one, five and ten seconds. Subsequently, 1  $\mu\text{L}$  of 0.5 mM trisodium citrate (Sigma-Aldrich, USA) solution was deposited onto the PET substrate in ten replicates as a model biological sample. The contact angles were measured by the instrument (See System E, Advex Instruments, Czech Republic) using a technique called "sitting droplet on a solid foundation". The device consists of a CCD camera placed on a sliding table in front of which a substrate plate is placed. Using a micropipette, 1 $\mu\text{L}$  droplet of sample is deposited onto the substrate and the See System software calculates the contact angle based on the interpolation of the height and width of the arc describing the drop shape. The size of the contact angle was evaluated immediately after modification of the substrate surface and further after one, seven and 31 days.

Based on the previous experiment, where the aging of the modified surface was monitored by varying the contact angle and the size of the stain, depending on the dropping time that elapsed from surface modification, a complementary experiment was performed. One mL of citrate sample was mixed with cadmium and indium standards (aqueous calibration solutions, CRM, Analytika, Czech Republic) so that the resulting solution contained 400  $\text{ng}\cdot\text{L}^{-1}$  of these standards. The 200 nL droplet sample solutions thus prepared were applied in five replicates using a micropipette on the surface of the modified PET substrate immediately, one, three and six days after the modification.

The Cd and In standards, chosen because of their similar weights and first ionization energies were used to correct the intensity of the monitored signals. The purpose of this experiment was to demonstrate whether the substrate aging affects the signals intensity. At the same time, the size of the stains was measured. Ideally, the application conditions should be the same as in the experiment where aging was observed by measuring contact angles. However, this was not possible due to

different sample volume requirements for the given measuring technique a volume of 1  $\mu\text{L}$  was required to measure contact angles and 0.2  $\mu\text{L}$  for SALD ICP MS.

The plate with samples was then inserted into the ablation cell (model UP213, New Wave Research, Inc., USA) equipped with a 213-nm Nd:YAG pulse laser, 3D positioning system and built-in CCD camera for visual control of the ablated samples. The dry aerosol created during ablation was analysed by the ICP mass spectrometer (Agilent 7500ce ICP-MS, Agilent, USA). In order to determine of the beginning and end of the ablation process, the  $^{13}\text{C}$  isotope was monitored in addition to the other isotopes of interest.

For the ablation of the stains, a "zig-zag" ablation trajectory was selected with a 170  $\mu\text{m}$  ablation line and a 160  $\mu\text{m}\cdot\text{s}^{-1}$  scanning speed. The remaining basic ablation parameters are shown in Tab.1.

*Table 1.* Ablation parameter settings

Isotope integration time	0.1 s
He carrier gas flow rate	1.0 $\text{L}\cdot\text{min}^{-1}$
Ar auxiliary gas flow rate	0.6 $\text{L}\cdot\text{min}^{-1}$
Ar plasma gas flow rate	15 $\text{L}\cdot\text{min}^{-1}$
Laser pulse repetition rate	10 Hz
Laser beam waist	250 $\mu\text{m}$
Laser fluence	$\sim 0.75 \text{ J}\cdot\text{cm}^{-2}$

## 3. Results and discussion

First, the contact angle formed by 1  $\mu\text{L}$  of 0.5 mM citrate droplets and PET substrate surface was evaluated immediately after modification of the substrate surface and then after one, seven and 31 days, the substrate surface being modified for one, five and ten seconds in ten replicates.

Fig.1 shows that the 0.5 mM citrate solution droplets that were applied onto substrate surface of PET modified by DCSBD for one second have the highest contact angle and hence less wettability; i.e., a longer duration of DCSBD modification causes a higher wettability. However, it is also evident from Fig.1 that the surfaces with the longest DCSBD modification times had the fastest tendency to return to their original state in the long term.



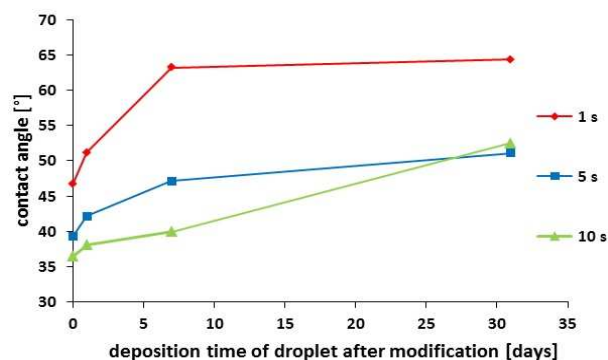


Figure 1. Dependence of the droplet contact angle size of 0.5 mM citrate solution against the PET surface modified by DCSBD for one, five and ten seconds; droplets were deposited immediately, one, seven and 31 days after the modification.

In the following experiments, the aging effect of the substrate surface modified by DCSBD on the intensity of SALD ICP MS signals was monitored. 200 nL droplets of 0.5 mM citrate solutions containing 400 ng.L<sup>-1</sup> of cadmium and indium standards were deposited in five replicates onto surface of the immediately modified PET surface and one, three and six days after modification.

From Tab.2, it is apparent that the stain sizes, which should be theoretically dependent on the wettability rate and the aging of the modified substrate surface, do not affect the repeatability of the assay for the given samples. Furthermore, it is evident that the average integrated intensity of the <sup>111</sup>Cd and <sup>115</sup>In signals from the stain ablation of the 0.5mM citrate sample immediately, one, three and six days after modification of the PET surface differed. This finding was against the assumption that these values should be the same, because in all cases the same amount of sample solution was deposited and the spots were practically 100 % ablated by visual inspection. The repeatability of the ablation was low and RSD values ranged above 30%. Such high values of RSD could be attributed, in particular, to the character of the sample itself, which was crystalline after drying, and the analytes as they were inhomogeneously distributed therein. However, when quantifying the signal intensity ratio of <sup>115</sup>In/<sup>111</sup>Cd, this inhomogeneity could be corrected, and the RSD values then varied around 7.5% for samples deposited immediately and one day after surface modification of the PET. The samples on the treated surfaces had the highest wettability and therefore, it was possible to assume

that the analytes were homogeneously dispersed in the stain. The ablation records in Figs.2A and 2B also show higher homogeneity of the stains. Samples applied three and six days after modification showed higher corrected values of RSD: 12 % and 19 % respectively, mainly due to a higher inhomogeneity (Figs.2C and 2D). Average <sup>115</sup>In/<sup>111</sup>Cd integrated signal intensity values for the spots applied one, three and six days after surface modification could be used for quantification of the monitored analytes as the values almost coincided. However, the average value of the integrated signal from droplets applied to the substrate immediately after the modification differed. This could be caused by extreme changes in the physical properties of the substrate surface immediately post modification.

Table 2. SALD ICP MS signals of <sup>111</sup>Cd and <sup>115</sup>In from stains of 0.5 mM citrate deposited at different times after PET surface modification.

Deposition time [days]	<sup>111</sup> Cd	<sup>115</sup> In	<sup>115</sup> In / <sup>111</sup> Cd	Stain size [mm]	
0	$\bar{X}$ [CPS]*	0,44	11	25	1,2±0,1
	RSD [%]	14	10	7,5	
1	$\bar{X}$ [CPS]*	1,4	31	22	1,6±0,2
	RSD [%]	34	38	7,2	
3	$\bar{X}$ [CPS]*	2,2	48	23	1,4±0,2
	RSD [%]	32	29	12	
6	$\bar{X}$ [CPS]*	2,8	58	22	1,2±0,3
	RSD [%]	34	24	19	

\*[CPS·10<sup>6</sup>]

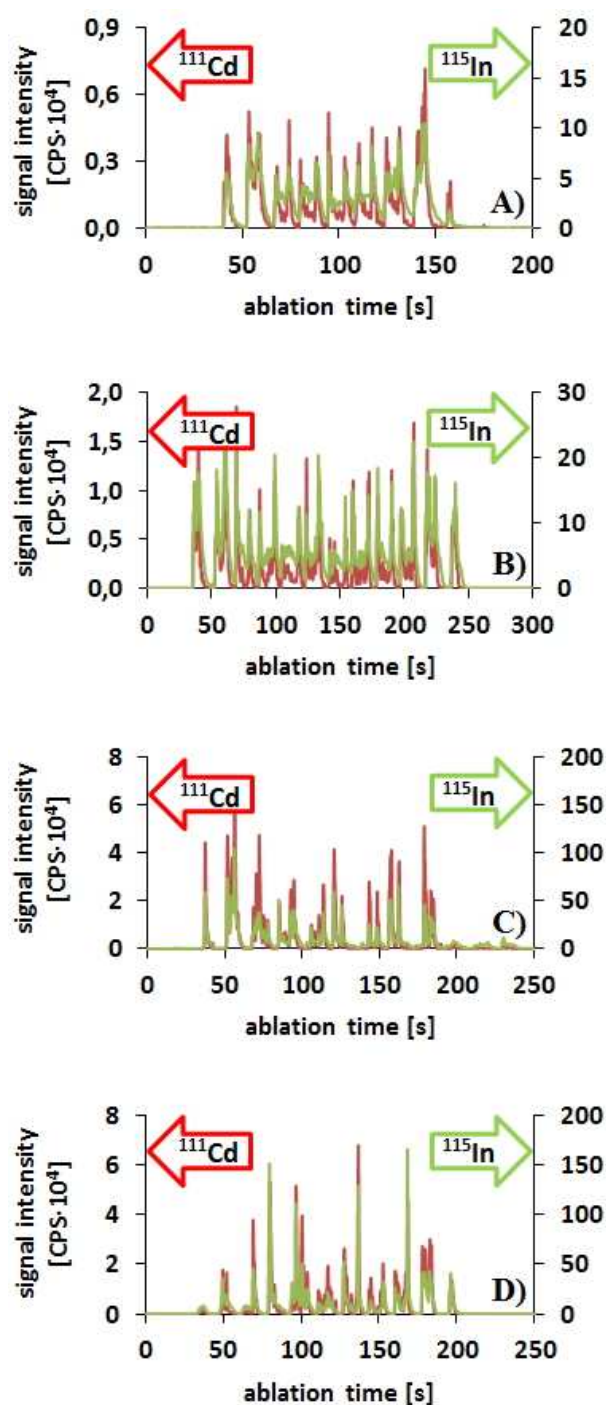


Figure 2. Ablation record of stains of 0.5 mM citrate deposited A) immediately, B) 1, C) 3 and D) 6 days after PET substrate surface modification.

#### 4. Conclusion

DCSBD technology was chosen for surface modification, i.e., increase of substrate surface energy and surface wettability. However, the disadvantage of this technique is the aging of the treated surface, i.e., the return of the modified physicochemical properties of the substrate surface

to its original state prior to the modification. The experiments showed that the integrated signal intensities of the selected analytes,  $^{111}\text{Cd}$  and  $^{115}\text{In}$ , present in the desorbed spots of 0.5mM citrate solution that were deposited at the substrate surface at various time intervals following its modification differed. Also, the repeatability of the analysis of individual samples expressed as RSD changes with deposition time. RSD values were around 30% for 0.5 mM citrate samples. When quantifying the ratio of integrated  $^{115}\text{In}/^{111}\text{Cd}$  signals, it was found that the values did not differ significantly from each other and could be used in quantitative analysis. Using internal standards, RSD values were reduced to less than 10 % for citrate samples.

#### Acknowledgement

We gratefully acknowledge the financial support of the Czech Science Foundation (GA 15-05387S) and the Ministry of Education, Youth and Sports of the Czech Republic under the project CEITEC 2020 (LQ1601).

#### REFERENCES

- [1] Peš, O.; Jungová, P.; Vyhnanek, R.; Vaculovič, T.; Kanický, V.; Preisler, J.: Off-Line Coupling of Capillary Electrophoresis to Substrate-Assisted Laser Desorption Inductively Coupled Plasma Mass Spectrometry. *Anal. Chem.* 2008 **80**(22), 8725–8732 DOI 10.1021/ac801036x
- [2] Navrátilová, J.; Jungová, P.; Vanhara, P.; Preisler, J.; Kanický, V.; Šmarda, J.: Copper ions regulate cytotoxicity of disulfiram to myeloid leukemia cells. *Int. J. Mol. Med.* 2009 **24**(5), 661–670 DOI 10.3892/ijmm\_00000277
- [3] Nehybová, T.; Šmarda, J.; Daniel, L.; Stiborek, M.; Kanický, V.; Spasojevič, I.; Preisler, J.; Damborský, J.; Beneš, P.: Wedelolactone Acts as Proteasome Inhibitor in Breast Cancer Cells. *Int. J. Mol. Sci.* 2017 **18**(4), 729 DOI 10.3390/ijms18040729.
- [4] Černák, M.; Černáková, L.; Hudec, I.; Kováčik, D.; Zahoranová, A.: Diffuse Coplanar Surface Barrier Discharge and its applications for in-line processing of low-added-value materials. *Eur. Phys. J.-Appl. Phys.* 2009 **47**(2), 1-6 DOI 10.1051/epjap/2009131
- [5] Štěpánová, V.; Kelar, J.; Galmiz, O.; Zemánek, M.; Slavíček, P.; Buček, A.; Černák, M.: Areal

homogeneity verification of plasma generated by diffuse coplanar surface barrier discharge in ambient air at atmospheric pressure. *Contrib. Plasm. Physic.* 2017 **57**(4), 182-189 DOI 10.1002/ctpp.201600093

[6] Prysiashnyi, V.: Atmospheric Pressure Plasma Treatment and Following Aging Effect of Chromium Surfaces. *JSEMAT* 2013 **3**(2), 138-145 DOI 10.4236/jsemat.2013.32018

## P22 THE POWER OF GU AND THE IMPORTANCE OF TEMPERATURE CONTROL

ANDRÁS GUTTMAN<sup>1,2,3</sup>, MÁRTON SZIGETI<sup>2,3</sup>, JEFF CHAPMAN<sup>1</sup>

1 SCIEX Separations, Brea, CA

2 Horvath Csaba Memorial Institute of Bioanalytical Research, University of Debrecen, Hungary

3 MTA-PE Translational Glycomics Group, University of Pannonia, Veszprem, Hungary

\*Correspondence: szigeti.marton@gmail.com

### Abstract

Analytical glycomics is a rapidly developing field due to the emergence of glycoprotein therapeutics and the growing interest of glyco-biomarker discovery. Glycans are among the most structurally diverse substances in nature, thus, their exact structural assignment represents a challenge. Capillary electrophoresis with laser induced fluorescent detection is one of the high-resolution analytical tools to address this issue, especially with automated glucose unit (GU) value calculation and the associated structural assignment using the built-in database. With the utilization of internal and co-injected bracketing standards, GU value calculation can be fully automated, and using the information from a single run supports highly accurate structural identification. It was observed, however, that separation temperature in capillary electrophoresis plays an important role in the migration properties of different carbohydrate structures manifested by shifting GU values and altered resolution. Thus, once a GU value database is established at a given temperature, all analyses should be conducted at that temperature to assure the adequacy of the automated structural assignment. Any changes in the separation temperature necessitate database modification, specific at that particular temperature. Since resolution between linear and even branched glycans can increase or decrease under different thermal conditions, temperature gradient capillary electrophoresis can be applied for selectivity optimization. Once the optimal separation temperature is defined for the glycan sample components in hand, all subsequent separations should be accomplished at that temperature with the associated database.

### Acknowledgment

The authors gratefully acknowledge the support of the Momentum (Lendulet) grant # 97101 of the Hungarian Academy of Sciences (MTA-PE Translational Glycomics) and the National Research, Development and Innovation Office (NKFIH) (K 116263) grants of the Hungarian Government. This work was also supported by the BIONANO\_GINOP-2.3.2-15-2016-00017 project.

## P23 DETERMINATION OF HOMOCYSTEINE IN URINE AND SALIVA BY MICROCHIP ELECTROPHORESIS

PETER TROŠKA,<sup>1\*</sup> ANNA MANDŽÁKOVÁ,<sup>1</sup> JASNA HRADSKI,<sup>1</sup> JURAJ ŠEVČÍK,<sup>2</sup> AND MARIÁN MASÁR<sup>1</sup>

<sup>1</sup> Department of Analytical Chemistry, Faculty of Natural Sciences, Comenius University in Bratislava, Mlynská dolina CH-2, Ilkovičova 6, SK-84215 Bratislava, SLOVAK REPUBLIC

<sup>2</sup> Department of Analytical Chemistry, Faculty of Science, Palacký University Olomouc, 17. listopadu 12, CZ-77146 Olomouc, CZECH REPUBLIC

**Abstract** – An on-line combination of isotachopheresis (ITP) and capillary zone electrophoresis (CZE) was employed on a microchip with a column-coupling technology for the determination of homocysteine in various body fluids. ITP with high concentration ability was used as a sample pretreatment and injection technique for CZE, which allowed rapid and sensitive determination of homocysteine. The resolution of the analyte from other constituents present in real complex samples was enhanced by discrete spacers, which were added to the injected sample. A solid phase micro-extraction pretreatment technique based on silver-form and barium-form resins was used prior to the ITP-CZE analysis to remove high concentrations of chloride and sulfate naturally present in the analyzed samples. The combination of the micro-pretreatment and micro-electrophoresis techniques allowed the determination of trace concentrations of homocysteine in urine and saliva samples.

**Keywords:** homocysteine, body fluids, microchip electrophoresis, solid phase microextraction

### 1. Introduction

Homocysteine (Hcy) is a non-protein-forming sulfur amino acid whose metabolism is at the intersection of two metabolic pathways: remethylation and transsulfuration. In remethylation, homocysteine acquires a methyl group from *N*-5-methyl tetrahydrofolate or from betaine to form methionine. The reaction with *N*-5-methyltetrahydrofolate occurs in all tissues and is vitamin B12 dependent, whereas the reaction with betaine is confined mainly to the liver and is vitamin B12 independent. A considerable proportion of methionine is then activated by ATP to form *S*-adenosylmethionine [1].

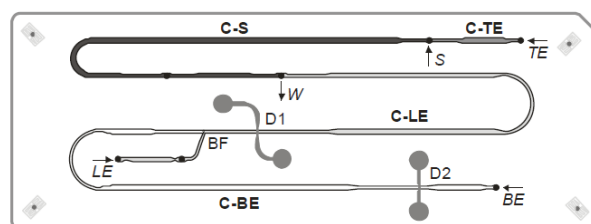
Increased concentration levels of Hcy in body fluids are considered to be an important risk factor or a marker of various diseases, particularly cardiovascular ones [2]. In review papers [3-5], individual methods of determination of Hcy in biological samples are summarized. Analytical methods using high performance liquid chromatography (HPLC) to separate Hcy are some of the most commonly used. HPLC methods for Hcy quantification utilize derivatization procedures as well as procedures with non-derivatized Hcy by electrochemical detection. The growing interest in clinical analyzes has generated increased attention to the rapid determination of Hcy using automated methods. For this reason, immunoassay of Hcy in plasma has become a preferred analytical approach [5].

Recently several procedures have been published for the determination of Hcy by capillary electrophoresis (CE). CE compared to HPLC is more advantageous in terms of the need for a very small sample volume, good resolution, short analysis time, simplicity of automation and elimination of various (toxic) solvents. This is evidenced by many of the works that dealt with the determination of Hcy in body fluids, e.g. in plasma [6-18], serum [13] or urine [16,19]. Most of them dealt with the use of laser-induced fluorescence as a detection technique, and UV detection was also used.

A miniaturized form of CE, microchip electrophoresis (MCE), was used for the separation and detection of Hcy and glutathione. The analysis time on

the glass microchip with amperometric detection was less than 80 s [6]. A PDMS microchip produced by a simple photolithographic technique allowed rapid separation of Hcy and cysteine [20]. MCE analyzes are more favorable than CE because they are considerably shorter, they achieve higher separation efficiency and, in particular, reduce overall costs associated with chemical consumption and waste production.

This paper deals with the development of new



**Figure 1.** A scheme of CC microchip with conductivity detection. C-LE = the first (ITP) separation channel (a 4.5  $\mu$ l volume) filled with leading electrolyte; C-BE = the second (CZE) separation channel (a 4.3  $\mu$ l volume) filled with background electrolyte; C-TE = the third channel (a 0.8  $\mu$ l volume) filled with terminating electrolyte; C-S = sample injection channel (a 9.9  $\mu$ l volume); D1, D2 = Pt conductivity sensors; BF = bifurcation section, BE, LE, TE, S = inlets for the background, leading, terminating and sample solutions to the microchip channels, respectively; W = an outlet channel to the waste container.

method for the determination of Hcy in various body fluids on the microchip with coupled channels (CC). The CC technology employed on the microchip with contact conductivity detection enables to couple on-line capillary zone electrophoresis (CZE) separations with isotachopheretic (ITP) sample pretreatment.

Urine and saliva samples were simplified by solid phase micro-extraction (SPME) prior to the ITP-CZE separations on the microchip. SPME based on silver-form and barium-form resins provided a high degree of compatibility with MCE and high selectivity, and at the

same time removed huge content of chloride and sulfate in the analyzed body fluids samples.

## 2. Experimental

ITP-CZE separations were carried out on a poly(methylmethacrylate) microchip (*Fig. 1*) with the CC technology and with integrated conductivity detection sensors (IonChip™ 3.0, Merck, Darmstadt, Germany).

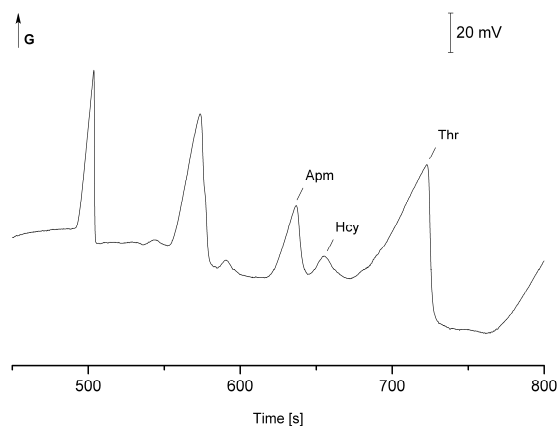
Chemicals used for the preparation of electrolyte solutions and model samples were obtained from Sigma-Aldrich (Bratislava, Slovakia), Fluka Chemika-BioChemika (Buchs, Switzerland), Serva (Heidelberg, Germany) and Lachema (Brno, Czech Republic). Samples of urine and saliva were collected from volunteers. Before analysis, the samples were homogenized and analyzed after appropriate dilution with deionized water, and pretreated by SPME to remove chloride and sulfate. SPE microcolumns of 0.5 mL volume containing silver-form and barium-form resins (Alltech, Grace Davison Discovery Sciences, Deerfield, USA) were used for this purpose.

## 3. Results and Discussion

ITP-CZE separations were performed in a hydrodynamically closed system with eliminated electroosmotic flow. These working conditions effectively reduce the fluctuations of total migration velocity of the analytes what is very important for reaching reproducible results, especially on the microchip with short separation paths [21]. In the ITP separations, leading electrolyte having a pH of 9.1 and glycine terminating electrolyte were used. In the CZE step of the ITP-CZE combination, the pH of the background electrolyte was 9.8.

The ITP realized in the first separation channel on the microchip (*Fig. 1*) preconcentrates the analyte and other sample constituents for the CZE separation step. On the other hand, close migration configuration of the constituents in the ITP stage of the ITP-CZE combination can be a limiting factor for reaching the required resolution of the analyte from matrix constituents, especially on the microchip with short separation channels. In such case, discrete spacers (DS) are effectively used to define the fraction of the sample transferred to the second CZE channel when the CC technology is employed. Then, sample constituents migrating outside of the mobility interval defined by the DSs are removed from the separation system. In this way, ITP works as an on-line sample clean-up technique prior to the CZE. In our case aminopimelate and threonine were used as the front and rear DS, and the undesirable sample components were electrophoretically removed from the separation system by column switching prior to the CZE separation realized in the second channel (*Fig. 2*).

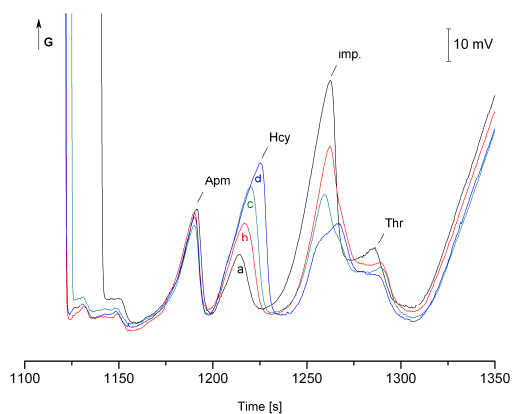
A  $1.4 \mu\text{mol l}^{-1}$  limit of detection (LOD) for Hcy was obtained by combination of ITP-CZE on the microchip. A relatively large sample volume injected on microchip ( $9.9 \mu\text{l}$ ) contributed to the reaching of low LOD value



*Figure 2.* Electropherogram from ITP-CZE separation of homocysteine in presence of discrete spacers. Sample injected on microchip:  $100 \mu\text{mol l}^{-1}$  Apm,  $15 \mu\text{mol l}^{-1}$  Hcy,  $150 \mu\text{mol l}^{-1}$  Thr in 50 % terminating electrolyte. Apm = aminopimelate; Hcy = homocysteine; Thr = threonine; G = conductivity.

when universal and not very sensitive conductivity detector was used. Under employed working and separation conditions, the reproducible migration velocities (RSD values of migration times were in the range 0.5 - 1.2 %) and determinations of trace concentrations of Hcy (RSD values of peak areas were 1.2 %) were achieved.

Considering high concentrations levels of chloride and sulfate in urine and saliva samples, these interfering anions were removed before ITP-CZE analysis from real samples by SPME using the resins in the silver and barium forms. The content of Hcy in body fluid samples was evaluated by the method of standard addition using ITP-CZE analysis after SPME pretreatment. The Hcy



*Figure 3.* Electropherograms from ITP-CZE analyses of urine sample after SPME pretreatment with various additions of Hcy. Sample injected on microchip: 2-times diluted urine,  $400 \mu\text{mol l}^{-1}$  Apm,  $200 \mu\text{mol l}^{-1}$  Thr in 50 % terminating electrolyte with (a) 0; (b) 10; (c) 20; (d)  $40 \mu\text{mol l}^{-1}$  Hcy. Apm = aminopimelate; Hcy = homocysteine; Thr = threonine; imp. = impurity; G = conductivity.



- homocyst(e)ine human plasma, *Electrophoresis*, 1999 **20**(3), 569-574
- [16] Liang, Q.; Chen, H.; Li, F.; Du, X.: Simultaneous sensitive MEKC-LIF determination of homocysteine, homoarginine, and six arginine metabolic derivatives in fluids from type 2 diabetics with peptic ulcer bleeding, *Chromatographia*, 2015 **78**(15-16), 1049-1056 10.1007/s10337-015-2919-6
- [17] Ševčíková, P.; Glatz, Z.; Tomandl, J.: Determination of homocysteine in human plasma by micellar electrokinetic chromatography and in-capillary detection reaction with 2,2'-dipyridyl disulfide, *J. Chromatogr. A.*, 2003 **990**(1-2), 197-204 10.1016/S0021-9673(03)00048-7
- [18] Chassaing, C.; Gonin, J.; Wilcox, C.S.; Wainer, I.W.: Determination of reduced and oxidized homocysteine and related thiols in plasma by thiol-specific pre-column derivatization and capillary electrophoresis with laser-induced fluorescence detection, *J. Chromatogr. B Biomed. Sci. Appl.*, 1999 **735**(2), 219-227 10.1016/S0378-4347(99)00425-9
- [19] Furmaniak, P.; Kubalczyk, P.; Głowacki, R.: Determination of homocysteine thiolactone in urine by field amplified sample injection and sweeping MEKC method with UV detection, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.*, 2014 **961**, 36-41 10.1016/j.jchromb.2014.04.051
- [20] Chand, R.; Han, D.; Islam, K.; Yeon, I.J.; Ko, S.S.; Kim, Y.S.: Microchip capillary electrophoresis based separation and detection of cysteine and homocysteine, *Adv. Mat. Res.*, 2013 **647**, 482-486 10.4028/www.scientific.net/AMR.647.482
- [21] Kaniansky, D.; Masár, M.; Bodor, R.; Žúborová, M.; Ölvecká, M.; Jöhnck, M.; Stanislawski, B.: Electrophoretic separations on chips with hydrodynamically closed separation systems. *Electrophoresis*, 2003 **24**(12-13), 2208-2227 10.1002/elps.200305474



# P24 ANALYSIS AND IDENTIFICATION OF POLYPHENOLIC COMPOUNDS IN GREEN FOODS USING A COMBINATION OF HPLC-ESI-IT-TOF-MS/MS

PAVEL ŠKVÁRA, JÚLIA KEZMANOVÁ, CSILLA MIŠLANOVÁ, ANDREA VOJS STAŇOVÁ

Department of Analytical Chemistry, Comenius University Faculty of Natural Sciences, Ilkovičova Bratislava, SK-842 15, Slovak Republic

**Abstract** – The beneficial effects of green foods on human health are well known. These benefits are mainly due to polyphenol content. The aim of this work was the development and utilization of advanced mass spectrometric (MS) technique combined with high performance liquid chromatography (HPLC) for analysis and identification of polyphenolic compounds in green foods. HPLC-ESI-MS/MS analyses of selected extracts prepared from various types of green food (*Urtica dioica* L. and *Allium ursinum*) and their polyphenolic profiles were performed by LCMS-IT-TOF analyzer equipped with electrospray ionization. HPLC separation were carried out on Kinetex XB-C18 (100 x 2.1 mm; 2.6 µm) using gradient elution (water+0.1% formic acid: acetonitrile+0.1% formic acid) with 0.2 ml/min flow rate. The MS1-MS3 analyses were performed within 50-1000 m/z range in both positive and negative ionization modes. Total analysis time was 20 minutes and injected volumes were 2 µl or 5µl, respectively.

**Keywords:** polyphenolic compounds, green food, HPLC-MS analysis, identification

## 1. Introduction

From a chemical point of view, antioxidants are phenolic compounds derived from simple phenols up to complex polyphenols. The antioxidant is a substance whose molecules limit the activity of oxygenated compounds - reduce their likelihood or lead them to a less reactive state. Antioxidants limit the oxidation process in the body or in the mixtures in which they are found. In food, they extend their expiration. In the body, they reduce the likelihood of certain types of diseases [1].

The main reason for monitoring the presence and activity of antioxidants is their beneficial effect on the human organism. Their health benefits include antioxidant, anti-bacterial, anti-carcinogenic, anti-allergenic and anti-inflammatory properties [2-4]. Antioxidants in green foods belong to a group of low-molecular-weight natural antioxidants whose scientific name is phenolic compounds [5]. Green foods are divided into four basic groups: herbs and spices, fruits, vegetables and nuts.

Despite the fact that numerous polyphenolic compound have been detected in the green food, there is still a need for new reliable analytical methods. The most common procedures for identification and determination of polyphenolic compounds in green food are based on sample pretreatment by liquid-liquid extraction followed by separation and identification using high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) [6]. Several authors are concerned with the

identification and characterization of phenolic compounds in green foods using a combination of high performance liquid chromatography and mass spectrometry with electrospray ionization (HPLC-ESI-MS) and atmospheric pressure chemical ionization (HPLC-APCI-MS). For example, HPLC-ESI-MS was used for separation and identification phenolic acids in custard apple [7], tannins in chestnut samples [8], phenolic compounds in groundnuts [9], tomato [10], spinach [11] and gingerol-related species (gingerols and shogaols) in ginger [12]. The HPLC-APCI-MS/MS method was used for identification of carotenoids in samples of mango and citrus [13]. The HPLC-ESI-MS method was used also to analyze samples of nettle [14] and bear's garlic [15].

The aim of this work was to analyze and identify polyphenolic compounds in selected samples of green food using a combination of high performance liquid chromatography and high resolution mass spectrometry techniques.

## 2. Experimental

All HPLC-ESI-MS/MS analyses were performed by using Shimadzu LC-MS-IT-TOF™ (Shimadzu, Kyoto, Japan). This mass spectrometer is combining an electrospray ionization (ESI), a 3D quadrupole ion trap (IT) and an orthogonally accelerated time-of-flight analyzer (TOF) as providing both the high sensitivity and high resolution of ions. The HPLC part consisted of: autosampler (SIL-20A), high pressure pumps (LC-

\*\*\*\*Correspondence: [skvara1@uniba.sk](mailto:skvara1@uniba.sk)

20AD), thermostat (CTO-20A), DAD detector (SPD-M20A), degasser together with mixer unit (DGU-20A5) and control unit (CBM-20A). LC-MS Solutions v 3.5.1. (Shimadzu) software was used for data acquisition and data evaluation.

Chromatographic separation of the polyphenolic compounds was performed on a Kinetex XB-C18 column (100 x 2.1 mm; 2.6  $\mu$ m), (Phenomenex, Torrance, CA, USA). Acidified water and acetonitrile (both with addition of 0.1 % or 10 mmol/L formic acid) were used as mobile phases. Gradient elution of mobile phase was used. The flow rate of the mobile phase was 0.2 ml / min. The gradient program was 0 min.: 5% B; 3 min.: 10% B; 8 min.: 40% B; 10 min.: 60% B; 11 min.: 90% B; 12 min.: 90% B; 12.1 min.: 5% B; 20 min.: 5% B. The chromatographic column was thermostated at 40 °C. All MS analyses were performed in both positive and negative ionization modes. The voltage at the ESI capillaries was +4.5 kV and -3.5 kV. The range of recorded values was set to 50-1000 m/z. The temperatures of the CDL capillary and heat block were set to 200 °C. The injected sample volume was 2  $\mu$ L or 5  $\mu$ L, respectively.

### 2.1. Chemicals

The following chemicals were used to prepare the solutions and mobile phases: water for LC-MS, acetonitrile for LC-MS (both LiChrosolv, Merck, Darmstadt, Germany), formic acid for LC-MS (Sigma-Aldrich, Steinheim, Germany). Standard of selected polyphenolic compounds were obtained from Sigma-Aldrich.

### 2.2. Sampling and sample preparation

The samples of the nettle (*Urtica dioica* L.) were collected in March 2017 in the geographical area of Hruštín, after which they were dried and stored in a dry and dark place. Samples of Bear's garlic (*Allium ursinum*) were collected in March 2017 in the geographical area of Bratislava, after which they were dried and stored in a dry and dark place. Prior to the analysis, the extracts of each sample were prepared as follows: Approximately 0.5 g of each was weighed and 50 ml water was poured at 97 °C. The extracting time was 5, 10 and 15 min. The extracts were then filtered through filter paper and cooled to room temperature. After cooling, the samples were filtered through a syringe microfilter (PVDF with pore size 0.22  $\mu$ m) and then analyzed

using HPLC-ESI-MS/MS or stored in a refrigerator at 4 °C for maximum of 24 hours.

## 3. Results and Discussion

The first part of the experimental work was focused on the optimization of conditions for chromatographic separation of phenolic compounds in selected extracts prepared from different plant species. After HPLC separation, optimization of the mobile phase composition with respect to the ionization of the analytes was performed. To confirm the effect of the mobile phase composition

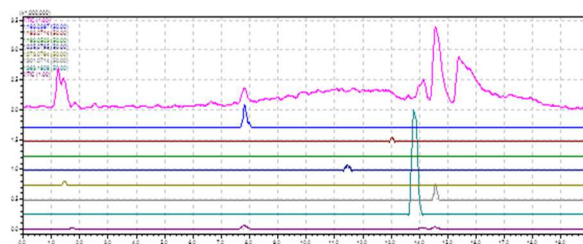


Figure 1. HPLC-ESI-MS analysis of the nettle extract (5 min. extraction) with the addition of 10 mmol/L formic acid to the mobile phases. TIC in positive mode - Top, TIC in negative mode - Bottom, Middle of figure including EIC records of identified phenolic compounds.

on the resulting MS signal,

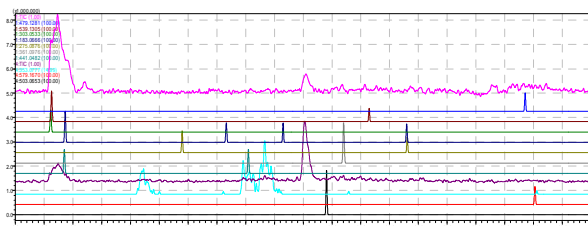
a simple experiment was proposed. HPLC-ESI-MS/MS analysis of the bear's garlic and nettle extracts used mobile phases, which varied only with the concentration level of the added formic acid. Gradient elution utilized, using the following mobile phases with different formic acid addition: 0.1% formic acid in water (A) / 0.1% formic acid in acetonitrile (B) and 10 mmol/L formic acid in water (A) / 10mmol/L formic acid in acetonitrile (B). For the illustrative purposes, *Figures 1 and 2* show the results from the HPLC-ESI-MS analysis of the 5 minute extract of the nettle. After comparing these results with the results of measuring a 5 minute nettle extract with the addition of 0.1% (26.5 mmol/L) formic acid to the mobile phases, it is clear that the use of a higher formic acid concentration is more appropriate for nettle extract analysis (promoting the protonation of molecules).

A similar procedure was used to select the appropriate composition of the mobile phases for

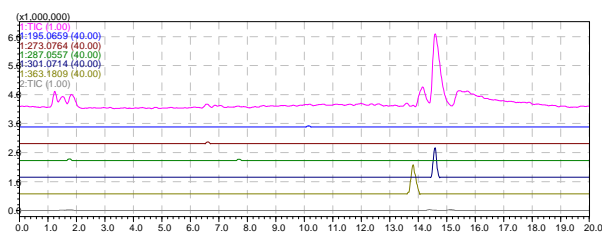
HPLC-ESI-MS analysis of the garlic extract. In *Figure 3* we can see the results of 15 minutes bear's garlic extract measuring with the addition of 0.1% formic acid to the mobile phases. *Figure 4* shows the results of 15 minutes garlic extract measuring with the addition of 10 mmol/L formic acid is preferable for the analysis of the garlic samples.

The final conditions for chromatographic separation and mass spectrometric detection are described in detail in the experimental part.

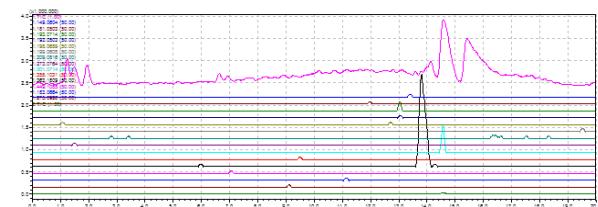
On the basis of the data obtained from HPLC-ESI-MS analyses, it is possible to obtain a better



*Figure 2.* HPLC-ESI-MS analysis of the nettle extract (5 min. extraction) with the addition of 0,1% formic acid to the mobile phases. TIC in positive mode TIC - Top, TIC in negative mode - Bottom, Middle of figure including EIC records of identified phenolic compounds.



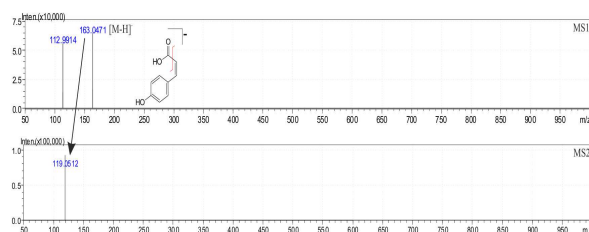
*Figure 3.* HPLC-ESI-MS analysis of the garlic extract (15 min. extraction) with the addition of 0,1% formic acid to the mobile phases. TIC in positive mode TIC - Top, TIC in negative mode - Bottom, Middle of figure including EIC records of identified phenolic compounds.



*Figure 4.* HPLC-ESI-MS analysis of the bear's garlic extract (15 min. extraction) with the addition of 10 mmol/L formic acid to the mobile phases. TIC in positive mode TIC - Top, TIC in negative mode - Bottom, Middle of figure including EIC records of identified phenolic compounds.

overview of the composition of individual extracts prepared from different plant species as well as information on the nature and structure of the phenolic compounds contained therein. Characterization and identification of individual polyphenolic compounds in plant extracts was performed by analysis of the obtained MS1-MS2 spectra.

In *Figure 5* we can see the MS1 and MS2 spectra of the coumaric acid in the sample of nettle extract. In the MS1 spectrum we see an ion with  $m/z$  163.0471 related to coumarone  $[M-H]^-$  ion. In the MS2 spectrum obtained by fragmentation of coumaric acid we see the ion with  $m/z$  119.0612, which was formed by cleaving the carboxyl group from the coumaric acid molecule. Another analyte found in the nettle sample was the chlorogenic acid, the presence of which was confirmed by the MS1 and MS2 spectra shown in *Figure 6*. The MS1 spectrum is dominated by the ion with  $m/z$  353.0780 corresponding to the  $[M-H]^-$  ion of chlorogenic acid. In the MS2 spectrum obtained by fragmentation of the ion with  $m/z$  353.0780 we see the ion with  $m/z$  191.0557, which was obtained by cleaving  $-C_9O_3H_7$  from the chlorogenic acid molecule. In this spectrum we further see the ion with  $m/z$  179.0378, which was formed by cleaving  $-C_7O_5H_6$  from the chlorogenic acid molecule. In MS2 spectrum we also see an ion with  $m/z$  173.0451, which corresponds to this cleavage. An ion with  $m/z$  135.0459 in MS2 seen in the chlorogenic acid spectrum was probably generated by cleaving the carboxyl group from the ion  $m/z$  179.0378.



*Figure 5.* MS1 and MS2 spectra corresponding to coumaric acid obtained by HPLC-ESI-MS/MS analysis of nettle extract (15 min. extraction) in the negative ionization mode.

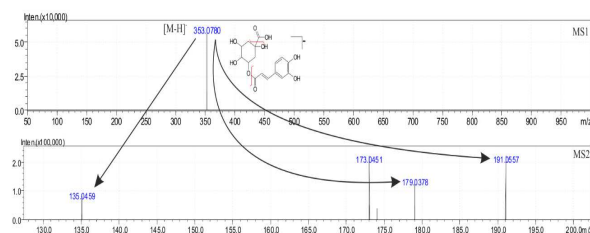


Figure 6. MS1 and MS2 spectra of the corresponding chlorogenic acid obtained by HPLC-ESI-MS/MS analysis of nettle extract (15 min. extraction) in the negative ionization mode.

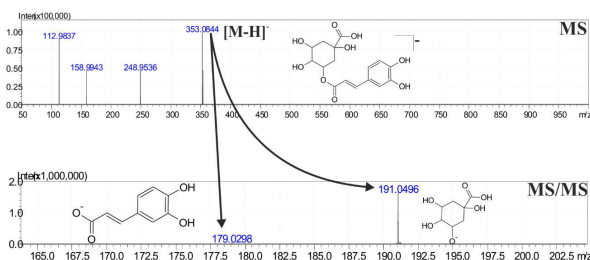


Figure 7. MS and MS/MS spectra corresponding to chlorogenic acid obtained by HPLC-ESI-MS/MS analysis of bear's garlic extract (10 min. extraction) in the negative ionization mode.

As in the case of the HPLC-ESI-HRMS analysis of nettle extracts, tandem mass spectrometry was used to identify polyphenolic substances in garlic extracts. For illustration, Figure 7 shows the MS1 and MS2 chlorogenic acid spectra. In the MS1 spectrum we see the molecular ion  $[M-H]^-$  with 353.0844 m/z, which is associated with the chlorogenic acid in a sample of the garlic extract. In the MS2 spectrum we see the ions with m/z 191.0496 and 179.0298 which were formed from the precursor ion (353.0844 m/z) and belong to the illustrated chlorogenic acid fragments.

The identification of each polyphenolic compound was possible via retention time as well as MS spectra of samples and authentic standards.

#### 4. Conclusion

In our experimental work, we focused on the use of advanced mass spectrometry techniques in combination with high performance liquid chromatography (HPLC-MS<sup>n</sup>) for the analysis and identification of polyphenolic compounds in extracts prepared from different types of green foods. From the HPLC-MS<sup>n</sup> data obtained by analysis of extracts from different types of green food, it was possible to obtain information about the

composition of the analyzed extracts as well as information about the nature and structure of the polyphenolic compounds represented therein. Using the developed HPLC-ESI-IT-TOF-MS method, we were able to confirm the presence of 13 antioxidants in the sample of nettle extracts and 16 antioxidants in the sample of garlic extracts. Based on the results obtained, it can be stated that the developed HPLC-MS<sup>n</sup> method is a suitable tool for the characterization and identification of polyphenolic compounds in extracts prepared from green foods.

#### Acknowledgement

The research was supported by projects VVCE-0070-07 and APVV-0583-11 of Slovak Research and Development Agency and GUK/321/2017.

#### REFERENCES

- [1] Gil, E. S.; Couto, R. O.: Flavonoid electrochemistry: a review on the electroanalytical applications, *Revista Brasileira de Farmacognosia*, 2013 23(3), 542–558 DOI:10.1590/s0102-695x2013005000031
- [2] Boros, B.; Jakabová, S.; Dörnyei, Á.; Horváth, G.; Pluhár, Z.; Kilar, F.; Felinger, A.: Determination of polyphenolic compounds by liquid chromatography–mass spectrometry in *Thymus* species, *Journal of Chromatography A*, 2010 1217(51), 7972–7980 DOI:10.1016/j.chroma.2010.07.042
- [3] Ginjom, I.; D'Arcy, B.; Caffin, N.; Gidley, M.: Phenolic compound profiles in selected Queensland red wines at all stages of the wine-making process, *Food Chemistry*, 2011 125(3), 823–834 DOI:10.1016/j.foodchem.2010.08.062
- [4] Atoui, A.; Mansouri, A.; Boskou, G.; Kefalas, P.: Tea and herbal infusions: Their antioxidant activity and phenolic profile, *Food Chemistry*, 2005 89(1), 27–36 DOI:10.1016/j.foodchem.2004.01.075.
- [5] Tsao, R.: Chemistry and Biochemistry of Dietary Polyphenols, *Nutrients*, 2010 2(12), 1231–1246 DOI: 10.3390/nu2121231
- [6] Orčić, D.; Francišković, M.; Bekvalac, K.; Svirčev, E.; Beara, I.; Lesjak, M.; Mimica-Dukić, N.: Quantitative determination of plant phenolics in *Urtica dioica* extracts by high-performance liquid chromatography coupled with tandem mass spectrometric detection, *Food Chemistry*, 2014 143, 48–53 DOI:10.1016/j.foodchem.2013.07.097

- [7] Baskaran, R.; Pullencheri, D.; Somasundaram, R.: Characterization of free, esterified and bound phenolics in custard apple (*Annona squamosa* L) fruit pulp by UPLC-ESI-MS/MS. *Food Research International*, 2016 82, 121–127 DOI:10.1016/j.foodres.2016.02.001
- [8] Comandini, P.; Lerma-García, M. J., Simó-Alfonso, E. F.; Toschi, T. G.: Tannin analysis of chestnut bark samples (*Castanea sativa* Mill.) by HPLC-DAD-MS, *Food Chemistry*, 2014 157, 290–295 DOI:10.1016/j.foodchem.2014.02.003
- [9] Ma, Y.; Kosińska-Cagnazzo, A.; Kerr, W. L.; Amarowicz, R.; Swanson, R. B.; Pegg, R. B.: Separation and characterization of phenolic compounds from dry-blanched peanut skins by liquid chromatography–electrospray ionization mass spectrometry, *Journal of Chromatography A*, 2014 1356, 64–81 DOI:10.1016/j.chroma.2014.06.027
- [10] Jiang, H.; Timmermann, B. N.; Gang, D. R.: Characterization and identification of diarylheptanoids in ginger (*Zingiber officinale* Rosc.) using high-performance liquid chromatography/electrospray ionization mass spectrometry, *Rapid Communications in Mass Spectrometry*, 2007 21(4), 509–518 DOI:10.1002/rcm.2858
- [11] Lucini, L.; Pellizzoni, M.; Baffi, C.; Molinari, G. P.: Rapid determination of lycopene and  $\beta$ -carotene in tomato by liquid chromatography/electrospray tandem mass spectrometry, *Journal of the Science of Food and Agriculture*, 2011 92(6), 1297–1303 DOI:10.1002/jsfa.4698
- [12] Dachtler, M.; Glaser, T.; Kohler, K.; Albert, K.: Combined HPLC-MS and HPLC-NMR On-Line Coupling for the Separation and Determination of Lutein and Zeaxanthin Stereoisomers in Spinach and in Retina, *Analytical Chemistry*, 2001 73(3), 667–674 DOI:10.1021/ac000635g
- [13] Petry, F. C.; Mercadante, A. Z.: Composition by LC-MS/MS of New Carotenoid Esters in Mango and Citrus. *Journal of Agricultural and Food Chemistry*, 2016 64(43), 8207–8224. DOI:10.1021/acs.jafc.6b03226
- [14] Farag, M. A.; Weigend, M.; Luebert, F.; Brokamp, G.; Wessjohann, L. A.: Phytochemical, phylogenetic, and anti-inflammatory evaluation of 43 *Urtica* accessions (stinging nettle) based on UPLC-Q-TOF-MS metabolomic profiles, *Phytochemistry*, 2013 96, 170–183 DOI:10.1016/j.phytochem.2013.09.016
- [15] Vlase, L.; Pârvu, M.; Parvu, A. E.; Toiu, A.: Phytochemical analysis of *Allium fistulosum* L. and *A. Ursinum* L, *Digest Journal of Nanomaterials and Biostructures*, 2012 8, 457–467

# P25 SILICA MONOLITHIC CAPILLARY COLUMNS FOR HILIC SEPARATIONS

DANA MORAVCOVÁ<sup>1</sup> AND JOSEF PLANETA<sup>1</sup>

<sup>1</sup> Czech Academy of Sciences, Institute of Analytical Chemistry, Veveří 97, Brno, 60200, CZECH REPUBLIC

The capillary monolithic columns (0.1 mm x 150 mm) prepared by acidic hydrolysis of tetramethoxysilane in the presence of polyethylene glycol and urea were modified to zwitterionic stationary phases and evaluated under HILIC separation conditions employing mixture containing nucleosides and nucleoside phosphates. The polymeric layer of zwitterion did not affect the high separation efficiency of original silica monolith. The prepared zwitterionic columns showed high separation efficiency exceeding values of 61 000 – 289 000 theoretical plates/m for 2-methacryloyloxyethyl phosphorylcholine-based stationary phase and 59 000 – 135 000 theoretical plates/m for [2- (methacryloyloxy)ethyl]-dimethyl-(3-sulfopropyl)-ammonium hydroxide-based stationary phase under optimal separation conditions. The grafted layer of zwitterion on the silica monolithic surface also significantly improved the separation selectivity to compounds of interest.

**Keywords:** HILIC, silica monolith, nucleoside, nucleotide, capillary chromatography

## 1. Introduction

Silica-based monolithic columns were developed by Nakanishi and coworkers in the early 1990s [1, 2]. Their preparation procedure was improved over the years [3]. The unique structure of silica-based monolith provides high permeability of separation bed and good mass transfer kinetics leading to high separation efficiency. Thus, they found the position in liquid chromatography as an alternative to particle packed columns.

While silica-based monolithic columns dedicated to reversed-phase separations are well established, the new stationary phases for HILIC separation are still under the research. Concerning the published papers, the preparation of silica-based monoliths via so-called “one-pot” process prevails. On the other hand, the chemical modification of preformed silica monolith offers well defined silica skeleton having almost the same permeability and separation efficiency for all designed stationary phases.

This contribution presents a characterization of silica-based monolithic capillary columns prepared employing the second approach. The zwitterionic monomers [2 - (methacryloyloxy)ethyl] - dimethyl - (3 - sulfopropyl) - ammonium hydroxide (MEDSA) and 2 - methacryloyloxyethyl phosphorylcholine (MEPC) were used to prepare desired stationary phases. The prepared columns were evaluated under HILIC separation conditions employing a mixture containing nucleoside and nucleoside phosphates.

## 2. Experimental

### 2.1. Material and Instrumentation

MEDSA and MEPC monolithic capillary columns were prepared following a protocol outlined in our previous study [4]. In order to obtain a stable polymeric layer of MEPC on the silica-based monolith, the polymerization time was prolonged to 6 hours.

Toluene, adenosine, uridine, cytidine, and nucleotides – adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), cytidine monophosphate (CMP), cytidine diphosphate (CDP), uridine monophosphate (UMP), and uridine diphosphate (UDP) were purchased from Sigma–Aldrich (Prague, Czech Republic). Acetic acid, ammonium hydroxide, and acetonitrile (ACN) (CHROMASOLV, HPLC gradient grade) were obtained from Riedel-de Haën (Prague, Czech Republic).

HPLC equipment consisted of two identical syringe pumps (100 DM with D-series controller, Teledyne Isco, Lincoln, Nebraska, USA) directly connected to a static nano mixer (60 nl volume) (Upchurch Scientific, Oak Harbor, WA, USA). The injection was performed by an electrically actuated E90-220 injection valve with 60 nl inner loop (Valco, Houston, TX, USA) and T-splitter with a restrictor (fused silica capillary 0.025 mm × 150 mm length). The inlet part of the monolithic column was inserted

Correspondence: moravcova@iach.cz

through the body of the splitter into the injection valve to minimize extra-column dispersion.

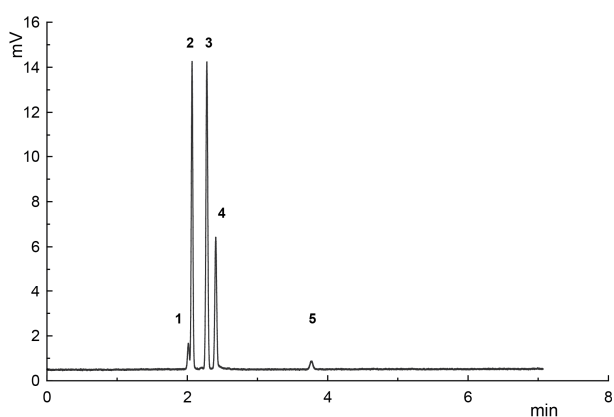
The monolithic column outlet was connected to a Spectra 100 UV–Vis detector (Thermo Separation Products, Waltham, MA, USA) via 0.035 mm × 120 mm long fused silica capillary (with optical bubble cell window i.d. 0.110 mm) using PTFE sleeve. UV detection was performed at 210 nm. The detector signal was processed by chromatography station software Clarity (DataApex, Prague, Czech Republic).

### 3. Results and Discussion

#### 3.1. Chromatographic properties of bare silica monolith

The prepared bare silica monolithic capillary columns were evaluated under HILIC conditions employing a mixture containing toluene as unretained compound and four purine bases such as xanthine, caffeine, theobromine, and hypoxanthine. *Fig.1* presents obtained chromatogram where all purine bases are baseline separated and have narrow symmetrical peaks.

The prepared column shows high separation efficiency reaching values of 135 000 – 220 000 theoretical plates/m corresponding to the minimum plate height in a range 4.5–7.4 μm, see *Table 1*.



*Figure 1.* HILIC separation of four purine bases on bare silica-monolithic capillary column. Mobile phase: 95% (v/v)ACN/5% (v/v) 100 mM ammonium acetate pH=4.5; flow rate 500 nL/min; UV detection at 210 nm. Peak identification: 1 –

#### 3.2. HILIC separation of nucleosides and nucleotides

*Table 1.* The retention factor ( $k$ ), the height equivalent to the theoretical plate ( $H$ ), the number of theoretical plates per meter ( $N$ ), and peak asymmetry ( $A_s$ ) obtained on silica monolithic column. See *Fig.1* for separation conditions.

Compound	$k$	$H$ [μm]	$N$	$A_s$
Toluene	-	7.4	135 185	0.67
Xanthine	0.03	4.5	222 547	0.88
Caffeine	0.13	5.8	172 795	1.00
Theobromine	0.19	5.2	192 528	1.13
Hypoxanthine	0.87	4.8	209 601	1.33

The commercially available methacrylate based monomers MEDSA and MEPC were selected for the preparation of zwitterionic stationary phases. The bare silica monolith prepared in 0.1 mm i.d. silica fused capillary was modified by 3-trimethoxysilylpropyl methacrylate and a polymer layer, with the appropriate zwitterion, was grafted to it. The suitability of these columns for isocratic HILIC separation of nucleosides and nucleotides was studied.

*Fig.2* presents chromatograms for selected compounds obtained on MEDSA (*Fig.2A*) and MEPC (*Fig.2B*) capillary columns. The better separation was obtained on MEPC column (*Fig.2B*) where all 11 compounds present in a sample mixture are well separated in a time less than 25 min. The value of peak asymmetry ( $A_s$ ) reflects the change in the molecular shape of the analyte and it slightly increases with increasing number of

*Table 2.* The retention factor ( $k$ ), the number of theoretical plates per meter ( $N$ ), and peak asymmetry ( $A_s$ ) obtained on MEPC column. See *Fig.2*. for separation conditions.

Compound	$k$	$N$	$A_s$
Toluene	-	138 955	1.0
Adenosine	0.16	201 048	1.0
Uridine	0.25	289 537	1.1
Cytidine	0.41	244 314	0.9
AMP	1.24	206 697	1.3
UMP	1.66	183 465	1.5
CMP	2.50	157 760	1.4
ADP	3.98	122 169	2.0
UDP	5.34	112 389	2.4
CDP	7.58	81 476	2.8
ATP	9.73	61 462	2.1

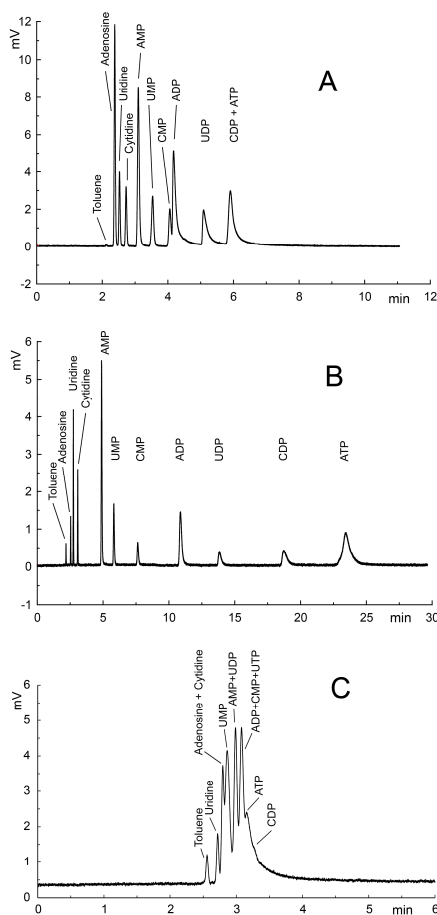


Figure 2. Separation of nucleosides and nucleotide phosphates on prepared MEDSA (A), MEPC (B), and bare silica (C) monolithic columns.

Mobile phase: 70% (v/v) ACN/30% (v/v) 50 mM ammonium acetate pH = 4.5; flow rate 500 nl/min; UV detection at 210 nm.

Table 3. The retention factor ( $k$ ), the number of theoretical plates per meter ( $N$ ), and peak asymmetry ( $As$ ) obtained on MEDSA column. See Fig.2 for separation conditions.

Compound	$k$	$N$	$As$
Toluene	-	131 000	1.2
Adenosine	0.12	135 641	1.1
Uridine	0.19	129 895	1.2
Cytidine	0.28	130 754	1.2
AMP	0.46	101 991	1.5
UMP	0.66	89 032	1.0
CMP	0.91	90 838	1.1
ADP	0.97	71 440	1.7
UDP	1.40	48 371	5.1
CDP	1.78	49 250	3.1
ATP	1.78	59 378	3.5

phosphate groups in relevant nucleotide, see Table 2.

On the other hand, the separation efficiency decreases with number of phosphate groups in the molecule of nucleoside phosphate and nucleotides show lower values of  $N$  compared to nucleosides. The similar trend was observed also on MEDSA modified monolithic capillary column.

MEDSA modified monolithic column shows lower separation efficiency than MEPC column for all nucleosides and nucleotides even the number of theoretical plates per meter is for toluene comparable, see Table 3. MEDSA column also shows lower retention for selected compounds, especially for di- and triphosphates of relevant nucleosides. This can be explained by the fact, that monomers used for column modification differ in their terminal groups, which affects the selectivity of the prepared stationary phases. The MEDSA monolithic capillary contains negatively charged end sulfo groups which decreases the retention of negatively charged nucleoside phosphates. On the other hand, the MEPC monomer provides to the stationary phase a slightly positive charge originating from the phosphorylcholine functional group which improves retention of negatively charged nucleoside phosphates under the HILIC separation conditions, e.g., the retention factor  $k$  for ATP is approximately 5-times higher on MEPC column comparing to MEDSA column.

The separation of nucleosides and nucleotides on bare silica monolithic capillary column shows Fig.2C. Bare silica monolith does not have sufficient selectivity to compounds of the target. All compounds are eluted out from the column in a time range cca 1 min which is accompanied with coelution of most compounds.

#### 4. Conclusion

Silica-based monolith in capillary format coated with zwitterionic polymer layers is a good alternative for analysis of complex mixtures containing highly polar compounds such as nucleosides and nucleotides. Especially, MEPC stationary phase containing positively charged terminal group enables to achieve the highly



selective HILIC separation of nucleosides and nucleotides. The utilization of such stationary phase to real sample analysis, where usually other highly polar interfering species originating from complex matrixes are present and complicate the analysis, should be evaluated in a future.

### Acknowledgement

This contribution has been supported by the Czech Science Foundation (Project No. 16-03749S), the Ministry of the Interior of the Czech Republic (Project No.VI20172020069), and by the Czech Academy of Sciences (institutional support RVO:68081715).

### REFERENCES

- [1] Nakanishi, K., Soga, N.: Phase separation in silica sol-gel system containing polyacrylic acid I. Gel formation behavior and effect of solvent composition, *J. Non-Cryst. Solids*, 1992 **139** 1-13 DOI: 10.1016/S0022-3093(05)80800-2
- [2] Minakuchi, H., Nakanishi, K., Soga, N., Ishizuka, N., Tanaka, N.: Octadecylsilylated Porous Silica Rods as Separation Media for Reversed-Phase Liquid Chromatography, *Anal. Chem.*, 1996 **68**(19) 3498-3501 DOI: 10.1021/ac960281m
- [3] Ikegami, T., Tanaka, N.: Recent Progress in Monolithic Silica Columns for High-Speed and High-Selectivity Separations, *ANNU. REV. ANAL. CHEM.*, 2016 **9** 317-342 DOI: 10.1146/annurev-anchem-071114-040102
- [4] Moravcová, D., Planeta, J., Kahle, V., Roth, M.: Zwitterionic silica-based monolithic capillary columns for isocratic and gradient hydrophilic interaction liquid chromatography, *J. Chromatogr. A*, 2012 **1270** 178 – 185 DOI: 10.1016/j.chroma.2012.11.005

# P26 MOLECULAR GLYCOHISTOPATHOLOGY BY CAPILLARY ELECTROPHORESIS

BOGLÁRKA DÖNCZŐ<sup>1,2</sup> AND ANDRÁS GUTTMAN,<sup>1,2\*</sup>

1 Horváth Csaba Laboratory of Bioseparation Sciences, Research Centre for Molecular Medicine, University of Debrecen, Nagyerdei krt. 98. Debrecen 4032 HUNGARY

2 MTA-PE Translational Glycomics Research Group, University of Pannonia, Egyetem u. 10, Veszprém 8200 HUNGARY

**Abstract** – N-linked glycans from standard glycoproteins, human serum and mouse tissue samples were investigated by capillary electrophoresis laser induced fluorescence (CE-LIF) detection. Intact, formalin treated and formalin-fixed, paraffin-embedded (FFPE) samples were used. FFPE samples were deparaffinized firstly, all of the samples solubilized with radioimmunoprecipitation assay (RIPA) buffer and digested with PNGase F endoglycanase enzyme for the N-glycan release. This step followed by fluorophore labeling and analysis by capillary electrophoresis. No significant differences were detected in the N-glycome profile at any investigated samples. The FFPE mouse tissue samples from lung have been used for N-glycan profiling. From this tissue sample, sixteen different N-linked carbohydrate structures were identified with exoglycosidase sequencing and GUcal software.

**Keywords:** FFPE, glycomics, capillary electrophoresis

## 1. Introduction

Since 1893, formalin was used for tissue fixation [1]. Furthermore, formalin fixation combined with paraffin embedment became the most prevalent method to preserve tissues from degradation [2]. This type of retained tissue is routinely prepared for pathological investigations, thus formalin fixed paraffin embedded (FFPE) tissue samples became almost exclusively used for long-term storage in histopathological laboratories and hospitals. A large number of archival tissue banks were established worldwide. FFPE specimens can serve as a valuable alternative for fresh frozen biopsy samples, which should be stored at -80°C [3].

The aim of our study was to develop a technique which could be suitable for global N-glycosylation profiling of FFPE tissues using high performance capillary electrophoresis analysis with laser induced fluorescence.

## 2. Experimental

### 2.1. Samples and Measurements

Fetuin, immunoglobulin G (IgG), ribonuclease B, human serum, radioimmunoprecipitation assay (RIPA) buffer, maltose, sodium cyanoborohydride, formaldehyde, paraffin and all other chemicals were from Sigma-Aldrich (St. Louis, MO). SCID male mice were used for the analyses. Animal-model protocols were carried out in accordance with the

Guidelines for Animal Experiments and were approved by the Institutional Ethics Committee at the National Institute of Oncology, Budapest, Hungary (permission number: 22.1/722/3/2010). PNGase F, Arthrobacter ureafaciens sialidase (ABS), Bovine kidney fucosidase (BKF), Jack bean galactosidase (JBG), Coffee bean  $\alpha$ -galactosidase (CBG) and Jack Bean hexosaminidase (JBH) were from ProZyme (Hayward, CA). The 8-aminopyrene-1,3,6-trisulfonate (APTS) and the maltooligosaccharide ladder were from SCIEX (Brea, CA).

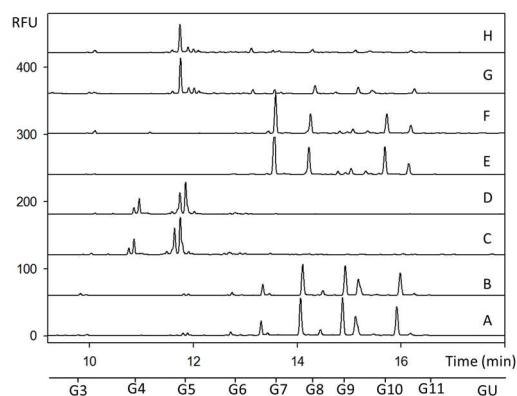
### 2.2. Theoretical Methodologies

Glucose Unit values of the peaks of interest are calculated by the GUcal software [4] ([www.gucal.hu](http://www.gucal.hu)) allowing adequate structural assignment using the built in database and the NIBRT based glycan database.

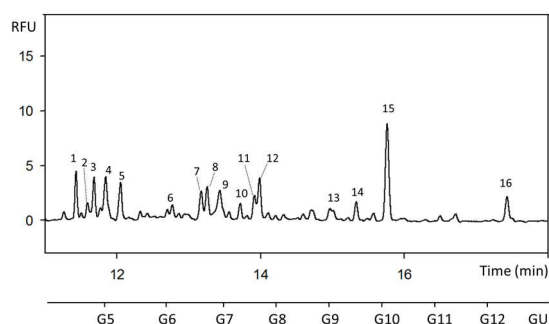
## 3. Results and Analysis

First the effect of formalin fixation and paraffin embedding were investigated on standard glycoproteins, human serum and mouse tumor tissue samples. Figure 1 represent the comparison of the APTS labeled N-glycan profiles of intact (A, C, E, G) and formalin-fixed, paraffin-embedded (B, D,

\*\*\*\*\*Correspondence: andras.guttman@hbs.org



**Figure 1.** CE-LIF traces of APTS labeled released glycans from IgG (A, B), fetuin (C, D), ribonuclease B (E, F) and human serum (G, H). Trace A, C, E and G: not treated control; trace B, D, F and H: formalin-fixed paraffin-embedded. Separation conditions: NCHO coated capillary, 50 cm effective length (60 cm total), NCHO separation gel-buffer,  $E = 500$  V/cm, temperature  $25^{\circ}\text{C}$ , Injection: 1 psi/5 s. The lower X axis shows the corresponding GU values.



**Figure 2.** CE-LIF profile of APTS labeled released glycans from FFPE mouse lung tissue sample. Separation conditions were the same as in Figure 1.

F, H) IgG (trace A and B), fetuin (trace C and D), RNase B (trace E and F) and human serum (Trace G and H). Y axis represents the relative fluorescent units (RFU). The two X axes show the migration time and the GU values of the APTS labeled maltooligosaccharide ladder which was used as standard for the experiments. Furthermore, APTS labeled maltose was injected with every sample as an internal standard for the trace alignment. No apparent differences were observed between the corresponding capillary electrophoresis traces. This result suggests that formalin fixation and paraffin embedment have no effect on the N-glycosylation of the investigated samples [5].

**Table 1:** Exoglycosidase array reaction mixtures

Exoglycosidase enzyme	a	b	c	d	e	f
ABS	-	+	+	+	+	+
BKF	-	-	+	+	+	+
JBG	-	-	-	+	+	+
CBG	-	-	-	-	+	+
JBH	-	-	-	-	-	+

**Table 2.** APTS-labeled mouse lung N-glycans, sequenced by exoglycosidase array and identified by GUCal software.

Peak#	MT [min]	GU	Structure
1	11.429	4.549	A2G(4)2S(6,6)2
2	11.588	4.721	A2G(4)2S(3,6)2
3	11.679	4.819	F(6)A2G(4)2S(6,6)2
4	11.842	4.996	F(6)A2G(4)2S(3,6)2
5	12.05	5.240	A1[3]G(4)2S(3)1
6	12.771	6.096	A2G(4)2S(6)1
7	13.171	6.607	F(6)A2[6]G(4)2S(6)1
8	13.254	6.713	M5
9	13.429	6.936	F(6)A2G1Ga1S1
10	13.727	7.326	F(6)A2[3]G(4)2S(6)1
11	13.913	7.595	M6
12	13.983	7.691	F(6)A2[3]G(4)2S(3)1
13	14.967	9.039	A2G(4)2
14	15.333	9.530	M8
15	15.762	10.106	F(6)A2G(4)2
16	17.433	12.366	FA2G2Ga2

Mouse lung tissue was used for the analysis, both fixed with formalin and embedded in paraffin. After the deparaffinization and the solubilization, the sample was released enzymatically, labeled with a charged fluorophore then analyzed by CE (Figure 2). The N-glycan pool of the lung tissue contains

mostly sialylated structures (GU=4-8) and some neutral (GU=9-13) oligosaccharides.

N-glycans from lung FFPE tissue were identified by their GU values during exoglycosidase array based carbohydrate sequencing. The reaction mixtures of the array contained *Arthrobacter ureafaciens* sialidase which remove  $\alpha(2-3,6,8)$  linked sialic acids; Bovine kidney fucosidase which release  $\alpha(1-2,3,4,6)$  fucoses; Jack bean galactosidase which remove  $\beta(1-4,6)$  linked galactoses; Coffee bean  $\alpha$ -galactosidase which cut all  $\alpha(1-3,4,6)$  galactose residues and Jack Bean hexosaminidase which remove the  $\beta(1-2,4,6)$  linked N-acetylglucosamines. 0.5 U was used from each exoglycosidase enzyme. The fluorophore labeled samples were digested at the same time. The digestion was incubated at 37°C overnight in 50 mM ammonium-acetate buffer (pH 5.5). The combinations of the reaction mixtures (a-f) are shown in Table 1. The ammonium-acetate content was removed by centrifugal vacuum evaporator drying.

Table 2 shows the glycan structures of the N-glycome of FFPE lung tissue which appeared in Figure 2. The calculation of the GU values was made by GUCal software. For the structural identification, publicly available databases were used as it was mentioned above [6].

#### 4. Discussion

All N-linked glycans of standard glycoproteins, human serum and mouse tissue samples were liberated by PNGaseF digestion before and after the fixation and embedding processes. The released glycans were labeled with fluorescent dye (APTS) and analyzed by capillary electrophoresis - laser induced fluorescent detection.

#### 5. Conclusion

Our study revealed that N-glycan profiles were identical before and after the formalin fixation and the paraffin embedding. It was demonstrated that FFPE samples could be useful for the identification of glycan structures which is the first step on the path to utilize huge sample collections located in hospitals for prospective and retrospective biomedical and biopharmaceutical studies.

#### Acknowledgement

The research was supported by the MTA-PE Translational Glycomics program (#97101), the NKFIH K116263 grant and the BIONANO\_GINOP- 2.3.2-15-2016-00017 project.

#### REFERENCES

- [1] Blum, F.: Formaldehyde, *Enzyklopadie d mikroskop Technik, Urban & Schwarzenberg* 1910 **1**(1): 478-92.
- [2] Fox, C.H.; Johnson, F.B.; Whiting, J.; Roller, P.P.: Formaldehyde fixation, *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* 1985; **33**(8):845-53.
- [3] Magdeldin, S.; Yamamoto, T.: Toward deciphering proteomes of formalin-fixed paraffin-embedded (FFPE) tissues, *Proteomics* 2012; **12**(7):1045-58.
- [4] Jarvas, G.; Szigeti, M.; Guttman, A.: Structural identification of N-linked carbohydrates using the GUCal application: A tutorial, *Journal of proteomics* 2017.
- [5] Donczo, B.; Szigeti, M.; Ostoros, G.; Gacs, A.; Tovari, J.; Guttman, A.: N-Glycosylation analysis of formalin fixed paraffin embedded samples by capillary electrophoresis, *Electrophoresis* 2015 **37** (17-18): 2292-6.
- [6] Donczo, B.; Szarka, M.; Tovari, J.; Ostoros, G.; Csanky, E.; Guttman, A.: Molecular glycopathology by capillary electrophoresis: Analysis of the N-glycome of formalin-fixed paraffin-embedded mouse tissue samples, *Electrophoresis* 2017; **38**(12):1602-8.

# P27 ENHANCED SEPARATION AND CHARACTERIZATION OF DEAMIDATED PEPTIDES WITH CE-MS

KLAUS FASERL,<sup>1</sup> BETTINA SARG,<sup>1</sup> AND HERBERT H. LINDNER<sup>1\*</sup>

<sup>1</sup> Division of Clinical Biochemistry, Innsbruck Medical University, Innsbruck, A-6020, AUSTRIA

Here we applied a low-flow capillary electrophoresis (CE) –electrospray ionization interface coupled to a mass spectrometer to analyze asparagine deamidation and aspartate isomerization. We achieved excellent resolution of asparagine (Asn), aspartic acid (Asp) and isoaspartic acid (iso-Asp) containing peptides using a synthetic peptide mixture. The migration order in CE enabled a clear assignment of in vitro deamidation/isomerization sites in a protein standard mixture of intermediate complexity (48 proteins) as well as the determination of the in vivo deamidation rate of histone H1.0 directly in a crude nuclear protein fraction.

**Keywords:** CE-MS, deamidation, iso-aspartate, isobaric peptides.

## 1. Introduction

Nonenzymatic asparagine (Asn) deamidation and aspartate (Asp) isomerization of proteins represents an important degradation reaction. Asn deamidation proceeds mainly through the formation of a succinimide ring intermediate that is quickly hydrolysed to n-Asp and isoAsp at a ratio of about 1:3. MS identification of deamidated peptides is relatively straightforward as deamidation adds 0.984 Da to the mass of a molecule. Analytical techniques for the identification and quantification of isoAsp are much more challenging primarily because Asp and isoAsp have identical mass and formal charge. Therefore, a reliable pre-separation is an important prerequisite for the analysis of deamidation products, as it adds complementary information before MS analysis.

## 2. Experimental

For CE-MS analysis a CESI 8000 (Sciex, Brea, CA) equipped with a bare fused or neutrally charged capillary (total length: 90 cm, i.d.: 30  $\mu$ m, o.d.: 150  $\mu$ m) was coupled via an ESI module to a Thermo Scientific Q Exactive Plus or LTQ Orbitrap XL ETD (Bremen, Germany). A synthetic peptide mixture consisting of six decapeptides containing Asn, Asp and isoAsp was used to examine the migration behavior on CE. In a next step, the Universal Proteomics Standard (UPS1) containing 48 human proteins was digested under different conditions to study the in vitro deamidation products and the reliability of CE-MS. Proteome Discoverer (ThermoScientific) with search engine

Sequest was used for data analysis. Raw data obtained by CE-MS from UPS1 were searched against the IPI human database version 3.86 (91,519 entries) to which 48 sequences of the Universal Proteomics Standard were added.

## 3. Results and Analysis

A synthetic peptide mixture consisting of Asn, Asp and isoAsp containing peptides was analysed using CESI-MS and a bare fused silica capillary. Optimization of the analysis conditions enabled a clear separation into six peaks (Figure 1). Identification of the isoAsp containing peptides was performed via ETD fragmentation, as this fragmentation method produces reporter ions (c+57 and/or z-57) which are unique to isoAsp. However, reporter ion intensity is often very weak and not all peptides are amenable for ETD fragmentation, such as short and low-charged peptide ions. Therefore, a reliable separation is an important prerequisite for the analysis of deamidation products. The change of net charge introduced by deamidation as well as the

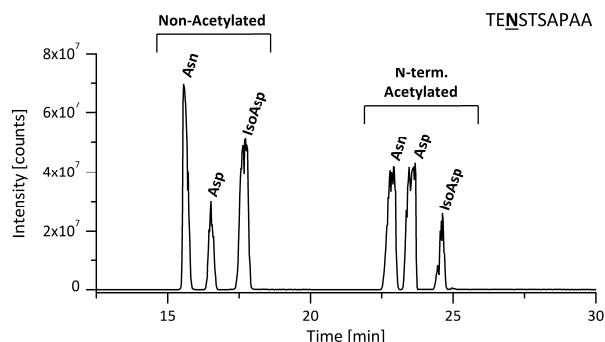
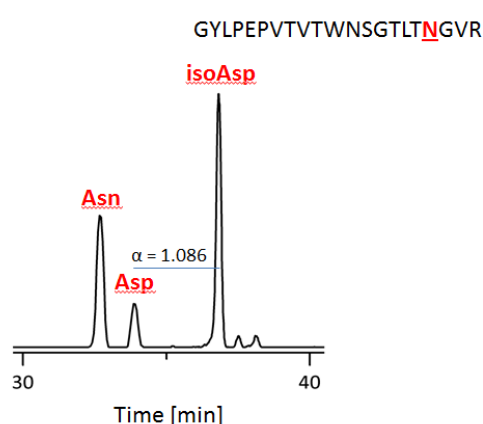


Figure 1. CESI-MS separation of 6 synthetic peptides covering deamidated and acetylated forms of the N-terminal peptide TENSTSAPAA of the human histone H1.0. CESI-MS conditions: Bare fused silica capillary; BGE, 0.1 % formic acid; MS instrument, Thermo Scientific LTQ Orbitrap XL ETD.

\*\*\*\*\*Correspondence: [Herbert.Lindner@i-med.ac.at](mailto:Herbert.Lindner@i-med.ac.at)

slight differences between the pKas of Asp and isoAsp makes CE particularly well suited for this kind of analysis. To improve the separation we replaced the bare fused silica capillary by a neutral coated capillary. In total, the CE-MS analysis of two digests yielded a total of 928 identified peptide sequences. A set of 390 peptides contained at least one asparagine and roughly one quarter was indeed found to be deamidated. On the basis of the migration times we were able to calculate the separation selectivity of the aspartate and isoaspartate containing peptides (Figure 2). The method enables an efficient separation of di-deamidated forms as well (Figure 2C).



*Figure 2.* Extracted ion electropherograms of the deamidated peptide GYLPEPVTVTWNSGTL-TNGVR present in the Universal Proteomics Standard. CESI-MS conditions: Neutrally coated capillary; BGE, 0.1 M acetic acid; MS instrument, Thermo Scientific Q Exactive Plus.

#### 4. Conclusion

Due to the reduced net charge introduced by deamidation as well as the slight differences between the pKa of the isomers Asp and isoAsp a reliable CE separation can be obtained. For further information on this topic we would like to refer readers to the full scientific publication on which this abstract to the CECE 2017 (14th International Interdisciplinary Meeting on Bioanalysis ) in Veszprém, Hungary is based[1].

#### Acknowledgement

The authors thank Jeff D. Chapman from SCIEX Separations for providing the sheathless high sensitive porous sprayer interface.

#### REFERENCES

- [1] Faserl K., Sarg B., Maurer V., Lindner H.H.: Exploiting charge differences for the analysis of challenging post-translational modifications by capillary electrophoresis-mass spectrometry. *J. Chromatogr. A.* 2017 **1498** 215-223 DOI: 10.1016/j.chroma.2017.01.086..

**Platinum Sponsor**



**Sponsors**



**BIO-SCIENCE**

[www.bio-science.hu](http://www.bio-science.hu)



kr<sup>o</sup>mat



Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

