CECE 2015

12th International Interdisciplinary Meeting on Bioanalysis

"… bringing people and ideas together …"

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Find the meeting history and more at www.ce-ce.org

Foreword

Welcome to CECE 2015, the 12th CECE conference in a row. While we still firmly stay behind the goal of "bringing together scientists who may not meet at specialized meetings, promote informal communication of researchers from different disciplines and map the current status of the fields shaping the bioanalytical science" we have made few minor changes in the program. First, the conference date had to be shifted earlier to avoid collision with other events. In addition the invited speaker's lectures predate the CECE Junior. The poster sessions will be open during all three days. This year the meeting is not free of charge anymore; however, we hope that the small registration fee will be more than compensated by the conference program. The organizers want to thank all invited speakers, sponsors and participants for their continuing support.

Brno, August 18, 2015

The Medal of Jaroslav Janák

The Medal of Jaroslav Janák for contributions to the development of analytical sciences was established by the Institute of Analytical Chemistry. Named after the inventor of the gas chromatograph (patented in 1952), founder of the institute (1956) and its long term director, the medal is awarded to scientists who have significantly contributed to the development of separation sciences.

This year the medal goes to **Professor Pavel Jandera**, a recognized analytical chemist, working at the University of Pardubice. Prof. Jandera is recognized for his research work in modern separation sciences, especially high-performance liquid chromatography. He has focused on the study of principles of separation, retention mechanisms, development, optimization and application of methods for the analysis of various types of substances in the environment, food and industrial products. His research interests include comprehensive theory of chromatography with programmed elution, and development of new methods for evaluation and characterization of columns for liquid chromatography. He has been also engaged in micro column liquid chromatography techniques, particularly in development of efficient monolithic capillary columns and two-dimensional liquid chromatography. The results achieved in this area were published in over 250 papers in scientific journals with more than 6000 citations. He also presented his research in over 250 lectures at foreign universities and conferences and served as a member of scientific committees of a number of international symposia in the field of analytical separations. Previously he has received the Tswett Foundation Award, Hanuš Medal of the Czech Chemical Society, Commemorative Medal of Nicolaus Copernicus University in Torun, Commemorative Medal of the University of Messina, Waksmundzki Award for important achievements in the field of separation sciences from Polish Academy of Sciences, Commemorative Medal of University of Pardubice, and in 2015 the AJP Martin Medal awarded by the UK Chromatographic Society.

Program - CECE 2015

Monday, September 21

8:00 – 15:00	**Registration**
9:00 – 9:30	**CECE 2015 - Opening remarks** **Presentation of the Jaroslav Janak's Medal to Prof. Pavel Jandera**
9:30 – 10:00	**ON-LINE MULTIDIMENSIONAL LIQUID COLUMN CHROMATOGRAPHY** **Pavel Jandera** *University of Pardubice, Pardubice, Czech Republic*
10:00 - 10:30	**ENGINEERING TUNNELS AND GATES IN ENZYMES** **Jiri Damborsky** *Loschmidt Laboratories, Masaryk University, Brno, Czech Republic*
10:30 – 11:00	**Coffee break**
11:00 – 11:30	**ELECTROKINETICALLY DRIVEN BIOANALYSIS IN MICROFLUIDIC SYSTEMS** **Adam T. Woolley, Radim Knob, Suresh Kumar, Vishal Sahore, Anna V. Nielsen, Mukul Sonker** *Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah, USA*
11:30 – 12:00	**IONIZATION MICROCHIPS IN MASS SPECTROMETRY** **Risto Kostiainen** *Division of Pharmaceutical Chemistry, University of Helsinki, Finland*
12:00 - 12:30	**LIVING DROPLETS – BIOMEDICAL DISCOVERY AT HIGH THROUGHPUT** **Christoph Merten** *European Molecular Biology Laboratory, Heidelberg, Germany*
12:30 – 14:30	**Lunch break – poster session**

14:30 – 15:00 **DIGITAL HOLOGRAPHIC MICROSCOPY: A NOVEL APPROACH FOR ASSESSING CELLULAR DYNAMICS IN REAL TIME**
Vratislav Kostal, Jan Balvan, Aneta Krizova, Tomas Slaby, Drahomira Ctvrlikova-Knitlova
Tescan, Brno, Czech Republic

15:00 – 15:30 **INORGANIC BIOACTIVE MATERIALS, BIOACTIVITY THERMODYNAMICS AND ASSOCIATED DENTAL USE OF TITANIUM**
Jaroslav Šesták
New Technology - Research Center in the Westbohemian Region, West Bohemian University, Pilsen, Czech Republic, and Institute of Physics, Academy of Sciences of the Czech Republic, Prague, Czech Republic

16:00 **City walk with Franta** – meet in the hotel lobby

19:00 **CONFERENCE DINNER WITH THE TRADITIONAL MORAVIAN MUSIC**

Tuesday, September 22

9:00 – 15:00 **Registration**

09:30 – 10:00 **ALTERNATIVE APPROACHES FOR SAMPLE PREPARATION IN CAPILLARY ELECTROPHORESIS**
Rosanne Guijt
University of Tasmania, Hobart, Australia

10:00 – 10:30 **BIOANALYTICAL STUDY OF THE BACTERIAL TRANSGLYCOSYLATION REACTION**
Bart Blanchaert, Erwin Adams, Ann Van Schepdael
KU Leuven, Leuven, Belgium

10:30 – 11:00 **Coffee break**

11:00 – 11:30 **INDUSTRIAL PRODUCTION OF INORGANIC AND POLYMERIC NANOFIBERS MADE BY FORCESPINNING TECHNOLOGY**
Jan Buk, Miroslav Tejkl, Jana Růžičková, František Foret, Jana Křenková
PARDAM s.r.o., Roudnice nad Labem, Czech Republic

11:30 - 12:00 **A LOOK AT CANCER UP CLOSE AND PERSONAL: THE ART OF LIQUID BIOPSY**
Marek Minarik
Genomac Research Institute, Prague, Czech Republic

12:00 – 14:00 **Lunch break – poster session**

14:00 – 14:30 **MULTIPLE HEART-CUTTING 2D-LC FOR ENHANCED QUANTITATIVE ANALYSES USING UV AND MS DETECTION**
Tom van de Goor
Agilent Technologies, Waldbronn, Germany

14:30 – 15:00 **IMPACT OF CHROMATOGRAPHIC CHANNEL GEOMETRY ON PERFORMANCE OF MICROFLUIDIC LC DEVICES**
Martin Gilar, Thomas S. McDonald, Bernard Bunner, Fabrice Gritti
Waters Corporation, Milford, MA, USA

15:00 – 15:30 **NOVEL MICROEXTRACTION TECHNIQUES IN PRETREATMENT OF COMPLEX SAMPLES**
Pavel Kubáň, Pavla Pantůčková, Andrea Šlampová, Petr Boček
Institute of Analytical Chemistry of the CAS, v. v. i. Brno, Czech Republic

15:30 – 16:00 **SEARCHING FOR GLYCAN CANCER BIOMARKERS: A COMBINED USE OF MASS-SPECTROMETRIC AND MICROCHIP CZE DATA**
Milos V. Novotny[1,2], William R. Alley, Jr.[1], Christa M. Snyder[1], Stephen C. Jacobson[1], Margit I. Campos[1]
[1]Department of Chemistry, Indiana University, Bloomington, Indiana, USA
[2]RECAMO, Masaryk Memorial Oncological Institute, Brno, Czech Republic

Program - CECE Junior 2015

Wednesday, September 23

8:55 – 9:00 **CECE Junior 2015 – Opening remarks**

9:00 – 9:30 **ELECTROCHEMISTRY OF BIOMACROMOLECULES AND ITS USE IN BIOMEDICINE**

Emil Paleček, Veronika Ostatná, Hana Černocká, Mojmír Trefulka, Vlastimil Dorčák, Veronika Vargová

Institute of Biophysics of the CAS, v. v. i., Brno, Czech Republic

9:30 – 9:45 **TILTED MICROPILLARS: A NEW ALTERNATIVE TO INCREASE MICROFLUIDIC CELL CAPTURE EFFICIENCY**

G. Járvás[1], I. Rajta[2], R. Huszánk[2], A.T.T. Szabó[2], G.U.L. Nagy[2], S. Szilasi[2], P. Fürjes[3], E. Holczer[3], Z. Fekete[3], M. Szigeti[1, 4], L. Hajba[1], J. Bodnár[1], A. Guttman[1, 4]

[1]MTA-PE Translational Glycomics Group, MUKKI, University of Pannonia, Veszprem, Hungary

[2]MTA Atomki, Debrecen, Hungary

[3]Hungarian Academy of Sciences, Centre for Energy Research, Institute of Technical Physics and Materials Science, Budapest, Hungary

[4]Horvath Csaba Laboratory of Bioseparation Sciences, University of Debrecen, Hungary

9:45 – 10:00 **CAPILLARY ELECTROPHORESIS-MASS SPECTROMETRY: AN EFFICIENT TOOL FOR MIDDLE-UP CHARACTERIZATION OF MONOCLONAL ANTIBODIES AND ANTIBODY-DRUG CONJUGATES**

Rob Haselberg[1], Klara Petru[2], Elena Dominguez Vega[1], Govert W. Somsen[1]

[1]Division of Bioanalytical Chemistry, VU University Amsterdam, the Netherlands

[2]Department of Analytical Chemistry, Faculty of Pharmacy in Hradec Kralove, Charles University Prague, Czech Republic

10:00 – 10:15 **QUANTUM DOT-BASED IMMUNOPROBE FOR OPTICAL AND ELECTROCHEMICAL DETECTION**

Veronika Dvorakova[1,2], Michaela Cadkova[1,2], Vladimira Datinska[3], Andrzej Chałupniak[4], Lucie Korecka[2], Arben Merkoçi[4], Karel Kleparnik[3], Frantisek Foret[3], Zuzana Bilkova[2]

[1]Department of Analytical Chemistry, Faculty of Chemical Technology, University of Pardubice, Pardubice, Czech Republic

[2]Department of Biological and Biochemical Sciences, Faculty of Chemical Technology, University of Pardubice, Pardubice, Czech Republic

[3]Institute of Analytical Chemistry of the CAS, v. v. i., Brno, Czech Republic

[4]Nanobioelectronics & Biosensors group, Institut Català de Nanociència i Nanotecnologia, Bellaterra, Spain

10:15 – 10:30 **NANOSTRUCTURED GOLD ELECTRODES FOR DETERMINATION OF GLUCOSE IN BLOOD**

Zdeněk Farka[1], Tomáš Juřík[1,2], David Kovář[1], Pavel Podešva[3], František Foret[1,3], Petr Skládal[1,2]

[1]CEITEC MU, Masaryk University, Brno, Czech Republic

[2]Department of Biochemistry, Faculty of Science, Masaryk University, Brno, Czech Republic

[3]Institute of Analytical Chemistry of the CAS, v. v. i., Brno, Czech Republic

10:30 – 11:00 **Coffee break**

11:00 – 11:15 **NOVEL PEMPDA β–CYCLODEXTRIN STATIONARY PHASE, STUDY OF ITS SEPARATION POTENTIAL**

Gabriela Kučerová[1], Květa Kalíková[1], Jindřich Jindřich[2], Eva Tesařová[1]

[1]Charles University in Prague, Faculty of Science, Department of Physical and Macromolecular Chemistry, Prague, Czech Republic

[2]Charles University in Prague, Faculty of Science, Department of Organic Chemistry, Prague, Czech Republic

11:15 – 11:30 **STRUCTURAL ANALYSIS AND RELATIVE QUANTIFICATION OF TIGHT JUNCTION PROTEINS: CLAUDIN-1 IN HUMAN SKIN BIOPSY USING CONFOCAL MICROSCOPE**

M. Svoboda[1,2], V.Pavlík[2], M.Hlobilová[2], T. Muthný[2]

[1]Department of Biological and Biochemical sciences, Faculty of Chemical-technology, University of Pardubice, Pardubice, Czech Republic

[2]Department of Research and Development, Contipro Biotech s.r.o., Dolní Dobrouč, Czech Republic

11:30 – 11:45 **COMPARISON OF MODELS USED FOR DESCRIPTION AND PREDICTION OF RETENTION BEHAVIOR OF OLIGOSACCHARIDES IN HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY**

Nikola Vaňková, Petr Česla, Jan Fischer

Department of Analytical Chemistry, Faculty of Chemical Technology, University of Pardubice, Pardubice, Czech Republic

11:45 – 12:00 **INFORMATION ENTROPY CALCULATION TECHNIQUE FOR THE DETECTION OF THE PHASES IN THE SELF-ORGANIZING REACTION**

Anna Zhyrova[1,2], Dalibor Štys[1,2], Tomáš Náhlík[1,2], Petr Císař[1,2]

[1]University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Centre of Aquaculture

[2]Biodiversity of Hydrocenoses, Institute of Complex Systems, Nové Hrady, Czech Republic

12:00 – 13:30 **Lunch break – poster session**

13:30 – 13:45 **PIECES OF KNOWLEDGE FROM THE STUDY ON THE ELECTROPHORETIC BEHAVIOUR OF SHORT OLIGODEOXYRIBONUCLEOTIDES IN FUSED SILICA CAPILLARIES**

Lada Vítová, Miroslav Fojta, Radim Vespalec

Institute of Biophysics, v. v. i., Academy of Sciences of the Czech Republic, Brno, Czech Republic

13:45 – 14:00 **LABEL FREE PROTEIN ANALYSIS AT CARBON ELECTRODES**

Veronika Vargová, Veronika Ostatná, Emil Paleček

Institute of Biophysics, Academy of Sciences of the Czech Republic, v.v.i., Brno, Czech Republic

14:00 – 14:15 **NOVEL POLYELCTROLYTES USED AS PHYSICALLY ADSORBED COATINGS – CAPILLARY ELECTROPHORESIS AND QUARTZ CRYSTAL MICROBALANCE STUDY**

Filip Duša[1], Joanna Witos[1], Erno Karjalainen[2], Tapani Viitala[3], Heikki Tenhu[2], Susanne K. Wiedmer[1]

[1]Department of Chemistry, University of Helsinki, Helsinki, Finland

[2]Laboratory of Polymer Chemistry, Department of Chemistry, University of Helsinki, Helsinki, Finland

[3]Centre for Drug Research, Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland

14:15 – 14:30 **COMPARISON OF IONISATION PROPERTIES OF AETMA-LABELED SACCHARIDES WITH COMMON LABELS**

Jan Partyka[1,2], František Foret[1]

[1]Institute of Analytical Chemistry of the CAS, v. v. i., Brno, Czech Republic

[2]Department of Chemistry, Masaryk University, Brno, Czech Republic

14:30 – 14:45 **ONLINE CONNECTION OF FREE-FLOW ISOTACHOPHORESIS CHIP TO AN ELECTROSPRAY IONIZATION MASS-SPECTROMETER**

Jukyung Park[1], Rosanne Guijt[2], Andreas Manz[1]

[1]KIST Europe GmbH, Saarbrücken, Germany

[2]School of Medicine and Australian Centre for Research on Separation Science (ACROSS), University of Tasmania, Hobart, Australia

14:45 **Closing remarks**

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P2 AN AUTOMATED CAPILLARY ELECTROPHORESIS IN A SEPARATION SYSTEM WITH ENHANCED SAMPLE LOADABILITY
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P3 EFFECTS OF COMPLEXATION OF BUFFER CONSTITUENTS WITH CHARGED CYCLODEXTRINS
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P4 COMPARISON OF ANTIOXIDANT AND COLOUR CHARACTERISTICS OF DIFFERENT TYPES OF MEDICAL PLANTS ASSESSED BY MODERN SPECTROSCOPIC TECHNIQUES
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P5 SYSTEM FOR FAST ANALYSIS OF EXPLOSIVES IN THE ENVIRONMENT
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P9 STUDY OF THE CELL WALL OF STAPHYLOCOCCUS AUREUS AND ITS SENSITIVITY TO ENZYBIOTICS
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Jan Tříska, Naděžda Vrchotová, Olga Vilímková

About the invited speakers

Pavel Jandera, PhD, DSc. (Prof. Ing. DrSc.) is professor of Analytical Chemistry, University of Pardubice, Vice-chairman: Group for Chromatography and Electrophoresis of the Czech Chemical Society, Member of the International Committee of the Central European Group for Separation Sciences, International Board of the "Mediterranean Separation Science Foundation Research", Editorial Board of the "Journal of Chromatography A" „Journal of Separation Science"Analytical Letters". Research fields: High Performance Liquid Chromatography (HPLC). Theory, prediction and optimization of HPLC separations, separation mechanisms, gradient elution, multidimensional separations, development of monolithic columns. Book: "Gradient Elution in Column Liquid Chromatography", >20 chapters in monographs, >250 research papers, >6000 citations, h-index = 44. Awards: Hanus medal of the Czech Chemical Society, memorial medals: University of Turun, Poland, University of Messina, Italy. Waksmundzki medal of the Polish Academy of Sience, M. Sklodowska medal of the Polish Chemical Society, A. J. P. Martin gold medal of the Chromatographic Society (London).

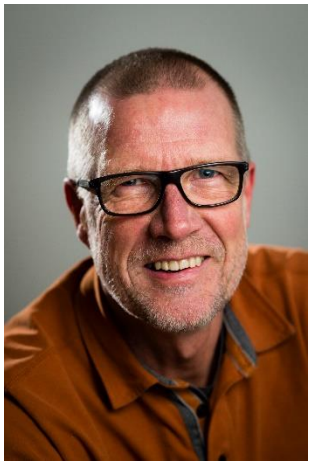
Jiri Damborsky is the Josef Loschmidt Chair Professor of Chemistry and Professor of Biochemistry at the Faculty of Science at Masaryk University in Brno, Czech Republic and a group leader at the International Centre for Clinical Research. Research of his group focuses on protein and metabolic engineering. His group develops new concepts and software tools for protein engineering (CAVER, HOTSPOT WIZARD, PREDICTSNP), and uses them for the rational design of enzymes and bacteria with improved properties for biocatalysis, biodegradation and biosensing. He has published >160 original articles, 12 book chapters and filed 6 international patents. He is a co-founder of the first biotechnology spin-off from Masaryk University Enantis Ltd. Among the awards and distinctions he has received is the award EMBO/HHMI Scientist of the European Molecular Biology Organisation and the Howard Hughes Medical Institute.

Adam T. Woolley graduated summa cum laude with a B.S. in Chemistry from Brigham Young University (BYU), Provo, Utah, USA in 1992. He received his Ph.D. in Chemistry in 1997 from the University of Californi - Berkeley under the direction of Professor Richard Mathies. His doctoral research involved the development of micromachined electrophoretic systems for rapid DNA analysis, and his work was recognized with the 1998 Fannie and John Hertz Foundation Thesis Prize. Woolley was a Cancer Research Fund Runyon-Winchell Foundation Postdoctoral Fellow in the group of Professor Charles Lieber at Harvard University from 1998-2000. His postdoctoral work focused on implementing carbon nanotube probes for high-resolution biological scanning probe microscopy. After postdoctoral studies, Woolley joined the Department of Chemistry and Biochemistry at BYU. He was promoted to Associate Professor in 2006 and to Professor in 2010. Prof. Woolley has also served as an Associate Department Chair since 2010.

Professor Woolley is author or co-author of more than 100 peer-reviewed papers, has given over 130 scientific presentations and has received 10 patents related to his work. He has received several recognitions, including the American Chemical Society Division of Analytical Chemistry Award for Young Investigators in Separation Science (2007), Presidential Early Career Award for Scientists and Engineers (2007), BYU Young Scholar Award (2008), BYU Reed M. Izatt and James J. Christensen Faculty Excellence in Research Award (2012), and BYU Karl G. Maeser Research and Creative Arts Award (2014).

The overarching theme of Professor Woolley's research is the interrelationship between biological molecules and miniaturization: he uses microfabrication techniques to create microfluidic systems to quantify clinically relevant biomolecules, and also utilizes biological molecules (in particular DNA) in designing and preparing nanoscale materials. He has trained over 30 undergraduate students, more than 20 graduate students, and 6 postdoctoral scholars in his group.

Woolley's current research is concentrated in three general areas: biotemplated nanofabrication, the creation of novel and sophisticated integrated microfluidic systems for enhanced biomarker quantitation, and the design of simple, miniaturized biomolecular assays. His group is developing ways to fold DNA into controlled nanoscale designs and convert these structures into functional nanomaterial systems through self-assembly and selective metallization. He is also combining affinity purification and solid-phase enrichment with electrophoretic separation in miniaturized devices to enable biomarker quantitation. Finally, his group is working to develop easy-to-use micro- and nano-fluidic chips for molecular analysis. These projects are pushing new frontiers in chemistry, medicine and engineering.



Risto Kostiainen
Professor, Vice Dean of Research Affairs
(risto.kostiainen@helsinki.fi)
Risto Kostiainen has acted as the professor of pharmaceutical chemistry at the University of Helsinki within the Faculty of Pharmacy since 1997. In addition he is acting as the Vice Dean of research affairs at the Faculty of Pharmacy. His research interest is focused to mass spectrometry, bioanalysis, microchip technology and separation sciences. The main application areas are metabolite analysis and metabolomics focused to brain research. Kostiainen has large national and international co-operation and he has headed and acted in several projects funded by the Academy of Finland, the Finnish Funding Agency for Technology and Innovation and European Union.

Christoph A. Merten (merten@embl.de) studied biochemistry at the University of Frankfurt and obtained his PhD on directed evolution of retroviruses at the Paul Ehrlich Institute in Langen, Germany. Subsequently he did a postdoc at the MRC Laboratory of Molecular Biology in Cambridge (UK), working on in vitro compartmentalization techniques. In 2005 he moved to the Institut de Science et d'Ingénierie Supramoléculaires (ISIS) in Strasbourg, France, where he became a junior group leader in 2007. Christoph joined the European Molecular Biology Laboratory in Heidelberg, Germany, as a Principal Investigator in 2010, focusing on microfluidic technology for HTS, diagnostics and genomics. His lab is particularly interested in the development of droplet-based microfluidics for cell-based screens. Christoph Merten is an inventor on a total of 13 patents (four of them with him as the sole inventor) and collaborates with many academic groups (e.g. The International AIDS Vaccine Initiative) and industrial companies (e.g. Diagenode, Fluidigm, Roche and GSK) in Europe, Asia and the US.

Vratislav Kostal is an applications specialist and a segment manager for Life Science at TESCAN, one of the leading manufacturers of scanning electron microscopes and focused ion beam systems.

Vratislav graduated with honors with M.S. in Environmental Chemistry from the Brno University of Technology, Czech Republic in 2003. In the same year, he joined the Institute of Analytical Chemistry, Brno, Czech Republic as a research assistant, where he was developing miniaturized fluorescence detectors for capillary separation methods. Vratislav received his Ph.D. in Analytical chemistry in 2007 from the Palacky University in Olomouc, Czech Republic. In 2007 he joined the laboratory of Prof. Edgar Arriaga at the University of Minnesota, Minneapolis, MN, USA. His postdoctoral work was focused on developing new technologies for the analyses of mitochondrial subpopulations using capillary electrophoresis and fluorescence microscopy. In 2012 Vratislav started his career at TESCAN, working as an Applications Specialist. Since 2015, he also works as a market segment manager for Life Science. In this new role, he oversees product support and marketing for TESCAN solutions dedicated to biotechnology and biomedicine, including correlative microscopy, cryotechniques and advanced FIB-SEM technology.

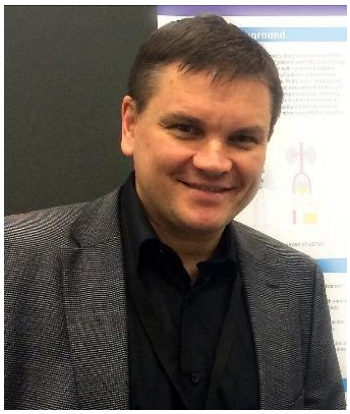
Jaroslav Šesták devoted his scientific proficiency in experimental and theoretical studies related to the fields of materials, applied thermodynamics and thermal analysis. As a full professor he has experience teaching not only in the field of material sciences and engineering but also in the interdisciplinary areas of philosophy and humanities. He edited and authored 14 books and monographs, published almost 300 papers (30 during the past five years) that have received about 2500 citations (Hirsh citation factor 24). Jaroslav gave over 150 invited key lectures and was presented with various scientific awards and assisted the underpinning of the School of Energy Sciences of Kyoto University (1996), Faculty of Humanities of Charles University in Prague (1999), Institute of Interdisciplinary Studies of West Bohemian University in Pilsen (2000) and Prague branch of the New York University (2000). Jaroslav was a co-founding member of both the ICTAC confederation (1965), Thermochimica Acta (1970), Journal of Mining and Metallurgy (1995) and recently the Global Journal of Analytical Chemistry (2010). Among important books belong his “Thermophysical properties of solids” (Elsevier, 1988); “Kinetic phase diagrams: nonequilibrium phase transitions” (Elsevier, 1991), “Special materials and their advanced technologies” (Academia, 1993), „Vitrification, transformation and crystallization of glasses“(Elsevier, 1996),

"Heat, thermal analysis and society" (Nucleus, 2004), "Science of heat and thermohysical study" (Elsevier, 2005), "Thermodynamics, structure and behavior of materials" (Pilsen 2009) and "Glassy, amorphous and nanocrystalline materials: Thermal physics, analysis, structure and properties" (Springer 2011 and "Thermal physics of micro-, nano- and non-crystalline materials (Springer 2013). Since 2010 is the doctor honoris causa of Pardubice University and a year later became the Emeritus Scientist of the Academy of Sciences. Beside his scientific career he was a league basketball player, mountaineer (Himalaya, Caucasus, Asian Pamir, South American Andes and the European Alps – earning needed funds as an occasional window-cleaner roping down tall buildings), ski instructor, politician (deputy and member of the Prague 5 government 1994-1998 and in 1996 a candidate for the seat in the Czech parliament) and enthusiastic globetrotter (notoriously carrying a sleeping sack in his backpack while participating at scientific conferences). Within this hobby he has also became a recognized photographer who held twenty three photo-exhibitions.

Rosanne Guijt completed her undergraduate degree in Biopharmaceutical Sciences at Leiden University, the Netherlands, and her PhD degree from Delft University of Technology where she worked between the Kluyver Institute for Biotechnology and the Institute de Microtechnique (IMT) at the Université de Neuchâtel in Switzerland. Her research involved the development of miniaturized total analytical systems (μTAS) for real-time monitoring of fermentations, and focused on the development of devices for capillary electrophoresis with integrated conductivity detection. She was awarded a fellowship from the Dutch Science and Technology Foundation STW to initiate Lab on a Chip research at the University of Tasmania (μTAS@UTAS). In Australia, she received a 4 year postdoctoral fellowship from the Australian Research Council to work in the Australian Centre for Research on Separation Science (ACROSS), and her research focus broadened to also include the development of flow-through microreactors. Her research strength is in the development of portable and field deployable analytical instrumentation, with applications in counterterrorism and environmental and bioprocess monitoring. She has also explored the use of 3D printing in microfluidics. Following her appointment in the School of Medicine focusing on the development of new technologies for point of care diagnostics and therapeutic drug monitoring. Currently, she resides at the Korean Institute for Science and Technology – Europe in Saarbrucken on an Alexander von Humboldt Fellowship for Experienced Researchers. She has published 67 articles in peer-reviewed journals with an average impact factor over 4.

Ann Van Schepdael (1964) obtained her diploma of Pharmacist in 1986, and PhD in 1990 (KU Leuven, pharmaceutical chemistry). Following a post-doc at KU Leuven and the Barnett Institute (Northeastern University, Boston, 1993) she was appointed as a lecturer in Leuven in 1997. Since 2007 she is a full professor at the Faculty of Pharmaceutical Sciences in the Pharmaceutical Analysis division, and heads the latter lab since 2010. Since 1990 her research focus is on analytical techniques, mainly capillary electrophoresis. One of her current interests lies in electrophoretically mediated microanalysis (EMMA) for enzyme studies and for chemical derivatization. She teaches courses on instrumental analysis and separation and absorption techniques, as well as practical pharmaceutical analytical chemistry exercises in the bachelor and master programs at the school of pharmacy KU Leuven. She has published over 200 publications in international peer-reviewed journals, has been the (co)-promoter of 25 PhD theses and is currently promoter of 4 PhD students and co-promoter of 4 PhD students.

Jan Buk – Chief Operating Officer, PARDAM s.r.o. at company focusing on Development and Production of Inorganic and Polymer nanofibers by Forcespinning technology. Member of the Executive Board of the Czech Association of Nanotechnology Industry. Member of the board of The Czech Society for Applied Photocatalysis. Experienced professional on the field of Development, Production and Application of nanofibrous materials with great experiences in technology Development, Commercialization and Project management at nanofiber industry.



Marek Minarik (1970) 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 40 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 DNA and RNA analysis with main emphasis on clinical cancer genomics.

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 funded partially from government research grants (domestic and EU). The center has developed own technology for screening and detection of molecular markers including liquid-biopsy technology for monitoring of cancer treatment and progression. Genomac is also an expert institute and a leading provider of DNA testing for medical and forensic genetics as well as direct to customer services such as genetic genealogy, traits and paternity testing.

Aside from commercial affiliation 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.

Tom van de Goor studied Chemical Engineering at Eindhoven University of Technology in The Netherlands where after obtaining his engineering degree (1987), he also completed his Doctor degree in Analytical Chemistry with Prof. Frans Everaerts, Prof. Carel Cramers and Prof. Pat Sandra in the field of Capillary Electrophoresis in 1992.

He then joined Hewlett-Packard in their Central Research Laboratories in Palo Alto, California. During this time he worked on and lead research teams in several technology fields related to micro scale separation technologies, such as capillary and chip based electrophoresis, low flow chromatography systems, HPLC-chip-MS, Time of Flight Mass Spectrometry and electrospray interfacing. Many of these have found their way into HP and Agilent Technologies products.

In 2002 he joined the Mass Spectrometry division within Agilent in Santa Clara, California where he lead teams both in R&D and Marketing in product and application development leading towards the introduction of the new 6000 MS series instruments in 2006. His specific technology focus was on ionization techniques such as nanospray, multimode ESI/APCI and API MALDI and applications focus in the Omics fields (Metabolomics and Proteomics) as well as small molecule Pharmaceutical development.

Since 2007 he is R&D section Manager at the Liquid Phase Separations Business in Waldbronn Germany, responsible for System Validation, Application and Research Collaborations for Chromatography systems and Capillary Electrophoresis and more focused on Pharmaceutical and Biopharmaceutical Analysis.

He leads the Agilent Core Technology University Relations (ACT-UR) Program in Europe, a grant program to support top research in areas of interest to the company and leads the German University relations program focused on

collaboration, instrument donation for teaching in separation science and talent search for internships and employee hiring.

Since 3 years he is teaching a Master Curriculum Course at the University of Marburg entitled: Bioanalytical Separation & Detection on Microchip Platforms.

He is author of more than 40 peer reviewed publications, 3 book chapters, 10 patents and (invited) speaker and contributor to over 100 International conferences and has been reviewer for numerous journals as well as the NIH and other grant agencies.

Martin Gilar is a principal investigator in Core Research group at Waters Corporation. He has more than 20 years of experience in the separation sciences, including chromatography, electrophoresis, and mass spectrometry. His research interest is analysis of biopolymers, and 2D LC. He has published over 40 peer reviewed papers.

Dr. Gilar's received his Ph.D. in analytical chemistry from Institute of Chemical Technology in Prague (1996). He spent postdoc years in Hybridon Inc. (1996-1998) and Northeastern University in Boston (1998) developing separation methods for antisense oligonucleotides and fraction collector for DNA molecules. Since 1998 he works at Waters Corp. in Milford, Massachusetts.

Pavel Kubáň has graduated in Chemistry and Mathematics at Masaryk University, Brno, Czech Republic in 1998, obtained his Ph.D. degree at Mendel University, Brno, Czech Republic in 2001 and RNDr. degree at Palacký University, Olomouc, Czech Republic in 2010. In 2003-2006 he worked as a postdoctoral fellow at University of Basel, Switzerland and spent 2 months in Australian Centre for Research on Separation Science (ACROSS) at University of Tasmania, Australia as a visiting scientist. Since 2006 until now he has been working at the Department of Electromigration Methods, Institute of Analytical Chemistry, Czech Academy of Sciences, where he is currently the head of the Department. His work is mainly devoted to capillary electrophoretic analysis of low-molecular weight compounds, to fundamental research and applications of novel microextraction techniques for pretreatment of samples with complex matrices and to direct coupling of these techniques to capillary electrophoresis. He is author or co-author of more than 70 scientific papers, reviews and book chapters and of nearly 40 contributions on international scientific conferences. In 2007 he was awarded Otto Wichterle Award (Czech Academy of Sciences) for outstanding young researchers.

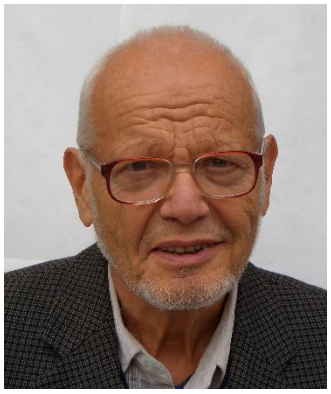
Milos Novotny has been a faculty member at Indiana University (Bloomington, Indiana, USA) for 43 years. He holds there the titles of Distinguished Professor and the Lilly Chemistry Alumni Chair. He is also an Adjunct Professor of Medicine and the Director of Institute for Pheromone Research. A native of Brno, Czech Republic, he received his undergraduate education and a doctoral degree in biochemistry at the University of Brno (now Masaryk University). Subsequently, Dr. Novotny held research appointments at the Czechoslovak Academy of Sciences in Brno (now the Institute of Analytical Chemistry of the Academy of Sciences) and the Royal Karolinska Institute (Sweden). He was a Robert A. Welch Postdoctoral Fellow at the University of Houston (under the direction of Albert Zlatkis) for two years.

He has been best known for his major role in developing modern chromatographic and electrophoretic methods of analysis. However, his general research interests are wide-ranging, including separation science and structural analysis of biological molecules, proteomics and glycoscience, and chemical communication in mammals. Dr. Novotny and his associates are known for structural identification of the first definitive mammalian pheromones. As a member of the Viking 1975 Science Team, Novotny designed the miniaturized GC column to search for organic molecules on the surface of Mars. He was a pioneer in the preparation of glass capillary columns for GC and coupling of capillary GC-MS during the late 1960s. A decade later, Novotny was responsible for the onset of the field of capillary LC, coming up with novel types of microcolumns, miniaturized detectors, and instrumentation. Capillary LC is now being routinely used under the names of "microflow LC" and "nanoflow LC" as an integral part of proteomics, lipidomics, glycomics, and metabolomics analytical platforms. Together with his former student, Milton Lee, Novotny was responsible for the renaissance of supercritical fluid chromatography during the 1980s. Milos Novotny made also major contributions to the development of capillary electrophoresis and capillary electrochromatography in the areas of protein, peptide and carbohydrate separations, including the design of unique fluorescent tags to assist these separations.

More recently, his group has been known for identification of disease biomarkers through glycomics and glycoproteomics. During his 43 years on the Indiana University faculty, Dr. Novotny has trained numerous students and visiting scientists who have become scientific leaders in separation science and bioanalytical chemistry, in both industry and academia.

Milos Novotny has authored over 500 journal articles, reviews, books and patents. He has received around 40 awards, medals and distinctions, including three honorary doctorates from European universities. His many awards include the American Chemical Society (ACS) Award in Chromatography (1986); the ACS Chemical Instrumentation Award (1988); the ACS Separation Science and Technology Award (1992); Eastern Analytical Symposium Awards in Separation

Science (1988) and Outstanding Achievements in the Field of Analytical Chemistry (2001), the Anachem Award (1992), the Dal Nogare Award (2004), the ACS Award in Analytical Chemistry (2005), and the Ralph N. Adams Award in Bioanalytical Chemistry (2008). Internationally, Dr. Novotny received the M. J. E. Golay Medal and was recognized by the Czech Academy (J. E. Purkynje Medal), the Russian Academy (M. S. Tswett Memorial Medal), the Royal Society of Chemistry of Great Britain (Theophilus Redwood Lectureship and the A. J. P. Martin Gold Medal) and Congreso Latinoamericano de Cromatografia Merit Medal (Argentina), and Giorgio Nota Award in Capillary Liquid Chromatography (2012) in Italy. He is a foreign member of two academies: The Royal Society for Sciences (Sweden) and the Learned Society of Czech Republic. Professor Novotny is a recipient of The J. Heyrovsky Honorary Medal for Chemical Sciences in 2015.



Emil Paleček received his PhD in biochemistry from Masaryk University in Brno, Czechoslovakia, in 1959. After working 5 years at the Institute of Biophysics of the Czechoslovak Academy of Sciences in Brno, he was a postdoctoral fellow with Professor Julius Marmur at the Graduate Department of Biochemistry, Brandeis University, Waltham, MA (1962-63). In 1967, he founded the Department of Biophysics of Macromolecules at the Institute of Biophysics in Brno and in 1969 he was promoted to Associate Professor. In 1989, he became a Corresponding Member of the Czechoslovak Academy of Sciences and in 1994 a Founding Member of the Learned Society of the Czech Republic. In 1993-1997, he was a Member of the Academy Council and in 2001-2005 and since 2013 a Member of the Scientific Board of the Academy of Sciences of the Czech Republic. He is Full Professor of Molecular Biology and Honorary Member of the Bioelectrochemical Society. In 2014 he was awarded a Medal of the Senate of the Parliament of the Czech Republic and the highest State award “The Czech Head” for his achievements in science. His research interests are in structure and chemical reactivity of nucleic acids and in electrochemistry of biomacromolecules and electrochemical biosensors.

Abstracts of oral presentations – Invited speakers

ON-LINE MULTIDIMENSIONAL LIQUID COLUMN CHROMATOGRAPHY

Pavel Jandera

Department of Analytical Chemistry, University of Pardubice, Pardubice, Czech Republic

Summary

The main objective of multidimensional (MD) chromatography is increasing the number of resolved compounds in a complex sample (peak capacity of the separation system). While off-line MD by TLC, GC and CLC techniques have been used for long time, on-line comprehensive technique where the whole sample is subject to separation in each dimension are relatively recent, especially as HPLC separations are concerned. The main issue in on-line two-dimensional (2D) separations is the necessity of accomplishing the second-dimension separation of the fraction collected from the first-dimension column in the short time (1-2 min or even less) available for the collection of the next fraction. For maximum peak capacity, different separation mechanisms should be used in each dimension of an MD system (RP, HILIC, Ion Exchange), however the mobile phases in each system should be compatible. For fast 2D separations, it is advantageous using gradient elution simultaneously in each dimension. The choice of optimum column combinations, dimensions and separation conditions (stationary and mobile phases, flow rate, fraction cycle time, etc.,) are discussed and examples of practical applications of on-line MD techniques are shown.

ENGINEERING TUNNELS AND GATES IN ENZYMES

Jiri Damborsky, Zbynek Prokop, Radka Chaloupkova, Jan Brezovsky

Loschmidt Laboratories, Department of Experimental Biology and the Research Centre for Toxic Compounds in the Environment, Masaryk University, Brno, Czech Republic
jiri@chemi.muni.cz

Summary

Protein structures contain a complex system of voids, making up specific features - clefts, pockets, cavities, channels and tunnels. These features are essential for the migration of solvents, ions and small molecules through the protein structure and

represent the natural hot spots for protein engineering. This migration is often controlled by highly dynamical structures called molecular gates. In this lecture, we will present: (i) examples of protein families possessing tunnels1 and gates2, (ii) software tools3 available for detection and analysis of tunnels and gates, (iii) success stories from engineering tunnels for catalytic activity4,5, enantioselectivity6 and stability7. We will demonstrate applicability of the software tools HOTSPOT WIZARD8 and CAVER9 for analysis and design of dynamical access pathways10 and will advocate the design of tunnels and gates as a powerful strategy for construction of novel biocatalysts. Moreover, we will also illustrate the importance of high-throughput bioanalytical techniques for directed evolution studies.

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ELECTROKINETICALLY DRIVEN BIOANALYSIS IN MICROFLUIDIC SYSTEMS

Adam T. Woolley, Radim Knob, Suresh Kumar, Vishal Sahore, Anna V. Nielsen, Mukul Sonker

Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah, USA

Summary

My research group is making micromachined devices that combine multiple analysis processes, which are used to quantify biomarkers linked to diseases. Our approach combines photopolymerized monolithic supports that carry out solid-phase affinity or reversed-phase extraction of target analytes, and microchip electrophoresis for separation and quantitation of selected components. In earlier work, we showed that electrically driven immunoaffinity extraction could be combined with microchip electrophoresis [1], and further, that solid-phase extraction and fluorescent labeling could be performed in monoliths in microfluidic devices [2]. We are utilizing both electrically driven and pressure-actuated methods for fluid manipulation within our microchips. We have recently developed microdevices with integrated pumps and valves for controlled injection of defined volumes of samples. These microchips also facilitate field-amplified stacking and injection of non-aqueous samples. We are now analyzing biomarkers relating to preterm birth in microfluidic systems that integrate immunoaffinity extraction, preconcentration, fluorescent labeling and electrophoretic separation. Our integrated microdevices have strong potential for broad application in studying biomarkers, especially where sample size is limited.

We are grateful to the United States National Institutes of Health (R01 EB006124) for partial support of this work.

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IONIZATION MICROCHIPS IN MASS SPECTROMETRY

Risto Kostiainen

Division of Pharmaceutical Chemistry, University of Helsinki, Finland

Summary

Miniaturization of analytical instruments utilizing micro-fabrication technology has been one of the hottest research topics in analytical chemistry over the past decade. Driving force to this is an increasing demand for low-cost instruments capable of rapidly analyzing very small amounts of samples with a high level of automation. A concept termed both “Miniaturized total analysis systems (μ-TAS)" and "Lab-on-a-chip" aims to develop integrated micro-analytical systems to perform complete analysis cycles (e.g. sample pre-treatment, chemical reactions, analytical separation, detection and data handling steps) on a single micro-device.

In the most micro-fluidic applications so far, on-chip detection has relied on optical detection, and for sensitivity reasons, fluorescence (FL) detection has been the most commonly utilized. Among the detection techniques alternative to optical detection, mass spectrometry (MS) has gained rapidly enhanced interest in chip-based analysis, and during the last few years great amount of reports have been published in the field. At present, the main focus is in integrating ionization methods to micro separation systems with MS.

Miniaturization of atmospheric pressure ionization techniques has gained rapidly enhanced interest in chip-based analysis. Electrospray ionization (ESI) is currently the method of choice to connect a microchip with mass spectrometry (MS). The flow rates used with microfluidic devices (nl-μl/min scale) are ideal for optimal sensitivity in ESI-MS. Different materials, such as silicon, glass, polymers have been used in fabrication of microchips. Recently SU-8 polymer has been shown to be highly suitable material for microfluidic separations and electrospray ionization.

Even though ESI is an excellent method for polar and ionic compounds, its sensitivity for neutral and non-polar compounds may be poor. Atmospheric pressure chemical ionization (APCI) and especially atmospheric pressure photoionization (APPI) offer alternative ionization techniques that is capable to ionize with high efficiency non-polar compounds. Recently we presented microchip APCI and APPI, which allow flow rates down to 50 nl/min making it directly compatible with microseparation systems. The chips provide excellent sensitivity, robust analysis, good reproducibility and cost efficient manufacturing. The feasibility of the APCI and APPI microchips in coupling of micro liquid chromatography (LC), gas chromatography and microchip LC to mass spectrometry is presented.

LIVING DROPLETS – BIOMEDICAL DISCOVERY AT HIGH THROUGHPUT

Christoph Merten

European Molecular Biology Laboratory, Heidelberg, Germany

Summary

We have developed fully integrated droplet-based microfluidic platforms for single-cell assays. In these systems tiny aqueous droplets (picoliter volumes) surrounded by oil serve as independent assay vessels. The technology allows the direct screening of >1 million primary, non-immortalized B-cells for the secretion of therapeutic antibodies. Furthermore, the technology can also be used for genomics applications and vaccine development, as we now show in collaboration with the International AIDS Vaccine Initiative.

DIGITAL HOLOGRAPHIC MICROSCOPY: A NOVEL APPROACH FOR ASSESSING CELLULAR DYNAMICS IN REAL TIME

Vratislav Kostal, Jan Balvan, Aneta Krizova, Tomas Slaby, Drahomira Ctvrlikova-Knitlova

TESCAN, Brno, Czech Republic

Summary

Cellular morphology and motility and their changes over time have been hallmarks of many cellular processes, so as a response to pathological processes, toxic stimuli and viral attacks. Invasive behavior of cancer cells can be also assessed by direct observations of cell proliferation.

Digital holographic microscopy (DHM) is an emerging microscopic technique for high resolution, label-free observations of living cells. Compared to standard microscopy, DHM has the unique ability to record complete information about the light waves passing through the sample. Intensity, DIC contrast and importantly phase shift can be easily computed from each hologram in real time. In particular, the phase shift images provide information of the mass distribution (i.e. thickness and shape) of the cells.

Here, we present the latest version of a multimodal holographic microscope (Tescan QPHASE) for long term studies of cellular dynamics and morphology. With the incoherent illumination setup, the system allows contrast imaging of cell boundaries with high clarity and excellent lateral resolution. Moreover axial sensitivity of phase shift detection enables observing very small changes in cell mass distribution. Due to the absence of halo-effect, images of cells can be easily

segmented, which is essential for reliable data analysis. In combination with the fluorescence module, the system becomes a comprehensive tool for investigating relationships between the morphological changes and involved biochemical processes.

The unique capabilities of the system are demonstrated by time lapse studies of cellular death, cancer cell behavior in 3D environments, and by observing dynamics of cancer development.

INORGANIC BIOACTIVE MATERIALS, BIOACTIVITY THERMODYNAMICS AND ASSOCIATED DENTAL USE OF TITANIUM

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Summary

Since 1970 two material groups have been identified capable to form a mechanically stable and functional interface with bone. One group consisted of certain soda-lime-silica glasses, which are exhibiting bone bonding ability (defined as "the bioactivity is the characteristics of an implant material which allows it to form a bond with living tissues"). Another material found to exhibit the bone–bonding ability was machined titanium. The phenomenon of attachment to bone was named osseointegration (defined as "osseointegration represents the formation of a direct contact of a material with bone without intermediate fibrous tissue layer, when observed using light microscope"). Apparently, surface quality determines tissue reactions to an oral implant and its assets can be classified regarding (i) mechanical (j) topographic (roughness, porosity, fractality) and (v) chemical properties. Bio-chemical bonding is related to bioactivity, which existence, however, has often been questioned because there is not a clear evidence of separated effects of surface roughness and interfacial chemical reactions. In modern dental and spinal implantology, advanced treatment protocols (e.g. early or immediate loading) are frequently used to enable reduction of the treatment time. A shorter healing period and shorter time of unloading, entails new demands on both the primary and secondary stability of the implant. The bioactivated surface, which is rich in hydroxyl groups, in contrast to machined surface, rapidly induces adsorption of calcium and phosphate ions on contact with the ions of the blood plasma. The calcium phosphate-rich layer promotes adsorption and concentration of proteins and constitutes a suitable substrate for the first apatite structures of the bone matrix, which are synthesized by the osteogenic cells at the beginning of the formation of the new bone tissue. The clinical study on dental implants was designed as a comparative study of two commonly used surfaces:

classical machined titanium surface and bioactivated titanium surface (LASAK©). The bio-surface is created by sand-blasting, acid etching and a final treatment in an alkaline solution andexhibits more favorable values of the major surface characteristics compared to the machined surface and other commercially available implant surfaces studied so far studied, such as the resonance frequency analysis method used to measure the implant stability quotient. A more easily wettable hydrophilic bio-surface allowed the contact formation between the body environment (blood) and the complicated rough and porous structure of the implant surface, and thus contributes to cell and bio-molecule migration and adhesion.

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ALTERNATIVE APPROACHES FOR SAMPLE PREPARATION IN CAPILLARY ELECTROPHORESIS

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Summary

In separation science, especially in capillary electrophoresis (CE), the process of sample preparation is often lengthier than the actual separation time and requires specialised equipment and trained personnel in a laboratory environment. Aiming to reduce analytical turn around times by bringing the analytical instrumentation to the sample, we developed a number of alternatives to commonly used sample preparation techniques. Because CE is instrumentally most compatible with the development of

portable and field deployable instrumentation, the focus was on the use of CE as separation technique.

First, the direct sample injection from fruits and vegetables will be presented [1]. Hydrodynamic effects caused by the separation capillary piercing the fruit were effectively excluded by increasing the hydrodynamic resistance of the separation capillary, enabling the electrokinetic injection from various fruits including zucchini, apple and mushroom. The analytical results correlated well with analysis by ICP-MS. Second, we developed a system for on-line monitoring of suspension cultures by sequential injection capillary electrophoresis [2]. Using only 8.1 mL of media (41 µL per run), the metabolic status and cell density were recorded every 30 minutes over 4 days. Thirdly, an electrokinetic size mobility trap was developed to selectively extract, concentrate and purify pharmaceuticals from whole blood [3]. Using nanojunctions of decreasing poresize, ampicillin, an antibiotic used for the treatment of sepsis, could be analysed from whole blood in less than 5 minutes.

With significant differences in their application, these examples demonstrate the feasibility of simplifying sample processing to enable fast and on-site analysis by CE with minimal human intervention.

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BIOANALYTICAL STUDY OF THE BACTERIAL TRANSGLYCOSYLATION REACTION

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Summary

Since the discovery of penicillin in the first half of the 20th century, antibiotics have contributed immensely to the improvement of general human health. However, soon after their mass introduction in medicine, the first signs of bacterial resistance towards these drugs have been noticed. Nowadays, the situation is alarming as more and more resistant strains are reported. One of the best known cases is the dangerous and

clinically important pathogen *S. aureus* of which 50 % of infections are caused by the MRSA strain which is resistant to all types of the β-lactam antibiotics. Only a few types of antibacterial therapeutics are left to treat patients infected with this strain. Therefore, research towards new antibacterial therapeutics is of the utmost importance. The bacterial cell wall and more specifically peptidoglycan, which already was the target of the first antibiotic, is still an interesting structure as not all processes involved in its production have been targeted.

The monomeric building block for the peptidoglycan is Lipid II. This is in essence a disaccharide unit to which a pentapeptide is attached, docked on an undecaprenyl pyrophosphate which serves as an anchor in the cell membrane. Lipid II is incorporated into peptidoglycan in two extracellular reactions. First the disaccharide unit is connected to a growing strand in a reaction called 'transglycosylation'. The undecaprenyl pyrophosphate anchor is disconnected in this step. In a second phase, transpeptidation, the cross-linking of saccharide strands by their pentapeptides, occurs after the linear glycan strands are formed. Both reactions are catalyzed by a group of membrane bound proteins called 'penicillin binding proteins' (PBPs). In contrast to the transpeptidation function of the PBPs, which is very intensively studied and is a well-known therapeutic target, the transglycosylation function has not led to any human therapeutic agents so far.

The study towards transglycosylation has been hampered by the unavailability of the substrate, Lipid II. Since Lipid II became available due to efforts in chemical and enzymatic synthesis about a decade ago, a wide variety of assays and high-throughput screens have been developed. The difficulty in the development of such an assay lies in the absence of a UV-chromophore in Lipid II causing the need for other alternatives, mostly radioactive or fluorescent labeling. Since work with radioactive labels is tedious and time-consuming and the attachment of fluorescent groups might alter affinity of inhibitors towards Lipid II, a label-free assay would be most interesting. Therefore, the goal of this project was to develop a label-free LC/MS assay for the bacterial transglycosylation reaction which could be used in a later stage to test inhibitors. In this study, PBP2 from *S. aureus* is used because it is the most important transglycosylase in this species. PBP2 had to be recombinantly produced and Lipid II was produced enzymatically. The development and validation of an LC/MS method for Lipid II will be described. In addition, the influences of the composition of the incubation mixture and incubation conditions on enzymatic activity have been evaluated and optimal conditions were selected. Under these circumstances, repeatable enzyme activity was obtained.

INDUSTRIAL PRODUCTION OF INORGANIC AND POLYMERIC NANOFIBERS MADE BY FORCESPINNING TECHNOLOGY

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Summary
Pardam a company located in the Czech Republic is focused on development and production of nanofibrous materials and products based on nanofibers. Pardam has great experiences with two different production technologies Electrospinning and Forcespinning. Both technologies are used for development of new nanofibrous materials as well as for mass production. There are two different types of nanofibrous materials in the product portfolio of the company. NnF CERAM – inorganic nanofibrous materials in powder like or cotton like structures and NnF MBRANE – polymeric nanofibrous membranes. Pardam has filed several patents and developed several commercial products based on nanofibers made by its technology. Presentation will be focused on introduction of Forcespinning technology, comparisons with Electrospinning, explaining challenges met during the technology development and optimization, on introduction of the final products based on nanofiber materials made by Pardam as well as sharing some experiences on post-treatment of nanofibrous materials in order to get them into the final product. Special attention will be dedicated to the phosphopeptide enrichment product based on inorganic nanofibers.

A LOOK AT CANCER UP CLOSE AND PERSONAL: THE ART OF LIQUID BIOPSY

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Summary
According to WHO in 2012 a total of 32.6 million people lived with cancer within 5 years from diagnosis (1). From the survival standpoint the most efficient therapy is an early surgical intervention in combination with efficient selection of other therapy modalities such as chemo-or radio- therapy. During the past decade the survival of some patients has significantly been prolonged with the arrival of targeted biological therapies. The effect of these new generation drugs is often directed by molecular signatures such as presence of specific somatic mutations within the tumor (2). The knowledge of tumors molecular makeup from tissue biopsy is therefore essential prior

to administering biological therapies. Histopathology evaluation of tissue biopsy has long been the primary tool in cancer diagnosis. More recently a testing for presence of molecular predictors (gene mutations or amplifications) from tissue biopsy samples has also become standard (3). The main problem for subsequent therapy decision comes when the tumor is either undetectable, inaccessible or the patient is incapable of undergoing an invasive procedure.

Short fragmented DNA has historically been observed in blood circulation of patients suffering from metastatic stages of cancers (4). Circulating DNA is often referred to as cell-free DNA to emphasize its exogenous nature in comparison to DNA originating from nuclei of the blood cells. Due to its exclusive origin in the cancerous cells, ctDNA retains the fundamental imprint of its cancer genome including cancer-specific aberrations such as somatic mutations. The most important uses include tumor diagnosis, early detection of tumor relapse or progression and, more recently, therapy prediction and survival prognosis (11). This new promising alternative, generally termed a "liquid biopsy", has immediately become one of the hot topics in cancer research (5). With liquid-biopsy the sampling can be done repeatedly with minimum invasivity. In addition to plasma, a former exploration of the ctDNA phenomena lead to its discovery inurine of patients with solid cancers (12). Subsequently, a transrenal passage of short DNA fragments has been verified offering an invaluable potential for further shift towards truly molecular cancer diagnosis involving repeated non-invasive sample acquisitions (6).

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MULTIPLE HEART-CUTTING 2D-LC FOR ENHANCED QUANTITATIVE ANALYSES USING UV AND MS DETECTION

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Summary

Two-dimensional liquid chromatography (2D-LC) has been demonstrated to greatly enhance separation performance compared to conventional 1D-LC. Using orthogonal separation mechanisms delivers much higher peak capacity than optimizing a one-dimensional method.

In contrast to full comprehensive 2D-LC, multiple heart-cutting (MHC) focusses on interesting regions of the 1D chromatogram and is predominantly used for targeted, quantitative analysis.

Factors influencing quantitation in 2D-LC using MHC approaches coupled to UV as well as mass spectrometry (MS) detection are discussed and examples of low level quantitation in complex matrices shown. The advantage of a multidimensional approach to approve LOQ in MS-quantitation obtained with strongly, electrospray-ionization suppressing matrix is also demonstrated.

The peak (heart-cut) parking functionality of MHC 2D-LC breaks the link between 1D and 2D time scales, which allows for addition of a second dimension to existing 1D-methods, the use of longer 2D-cycle times and columns with higher separation efficiency, and the use of 2D-flow rates that are acceptable to mass spectrometry detection.

IMPACT OF CHROMATOGRAPHIC CHANNEL GEOMETRY ON PERFORMANCE OF MICROFLUIDIC LC DEVICES

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Summary

Microfluidic LC is promising alternative to conventional scale chromatography especially for high sensitivity applications. However, chromatographic efficiency of microfluidic LC devices (tiles) packed with sub two micron sorbents is often lower compared to equivalent UHPLC columns. This is in part due to extra column band dispersion and in part due to the channel geometry of microfluidic tile columns adding to column dispersion. We evaluated the impact of turns in the chromatographic bed (including right angle turns at the entry and exit points of planar tile) on chromatographic performance of 0.3 and 0.5 mm ID's microfluidic tiles. The

measurement was performed using optimized LC system with minimal pre-column (sample injector) and post-column (detector cell) band dispersion. Direct efficiency measurement for 0.5 and 0.3 mm ID straight tiles showed that their efficiency is comparable to 2.1-mm ID UPLC columns. However, a distinct loss of efficiency was observed for tiles that included turns in the chromatographic bed. We optimized the tile geometry (turn radius, turn tapering) to improve the tile performance. Computational fluidic dynamic modeling using COMSOL software was used to predict the impact of tile geometry on efficiency and to improve the tile design. Predicted losses in efficiency were in a good agreement with experimentally measured data.

NOVEL MICROEXTRACTION TECHNIQUES IN PRETREATMENT OF COMPLEX SAMPLES

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Summary
Fundamental considerations and practical applications of various microextraction techniques for pretreatment of samples with complex matrices are summarized in this contribution. Special emphasis is devoted to electrically driven microextractions, such as electromembrane extraction (EME) across supported liquid membranes (SLMs), their down-scaling to micro- and nano- format and their applications in analyses of biological samples. New phase interfaces, such as free liquid membranes (FLMs) and polymer inclusion membranes (PIMs) are described and their potential for microextractions of undiluted biological samples is demonstrated. Various approaches for in-line coupling of the presented microextraction techniques to home-made and commercial capillary electrophoresis (CE) instrumentation are also presented. Full automation of the entire process, low instrumental requirements, low costs of the developed microextraction devices and membranes, their disposability and other aspects make the application of such hyphenated microextraction/CE techniques very attractive for routine clinical analyses.

1 Introduction

Analysis of complex, particularly biological, samples is burdened by their inherent properties. Matrix of biological samples, such as human blood, is highly complex and contains large quantities of salts, proteins, lipids and fatty acids. These matrix components usually adhere onto the inner surface of analytical instrumentation and result into deteriorated analytical performance and/or analytical system poisoning.

Standard extraction techniques, such as liquid-liquid extraction (LLE) and solid-phase extraction (SPE), are usually employed to eliminate the detrimental effects of matrix components. In academia, LLE and SPE have been recently replaced with micro-scaled sample pretreatment techniques [1,2]. Liquid-phase microextraction (LPME) [1] and solid phase microextraction technique [2] are most frequently used these days. In this contribution, novel LPME approaches will be presented, which were developed in our laboratory with special emphasis on simplification of sample pretreatment, reduction of sample volume and extraction time and on full automation of the entire microextraction/analytical procedure.

2 Experimental

Instrumental equipment and basic operational principles for EMEs, micro-electromembrane extractions (μ-EMEs) and extractions across SLMs, PIMs and FLMs were described previously [3-7].

3 Results and Discussion

Standard EMEs are performed with mL volumes of body fluids. Extractions of mL volumes of body fluids may, however, be complicated in certain cases due to their limited availability. Concept of a down-scaled μ-EME was proposed recently, where nL to μL volumes of body fluids can be extracted across FLMs in transparent perfluoroalkoxy (PFA) tubing [4]. Figure 1A depicts the basic instrumental arrangement of μ-EME across FLM and Figure 1B demonstrates applicability of such arrangement in μ-EMEs of basic drugs from 1.5 μL of undiluted human body fluids.

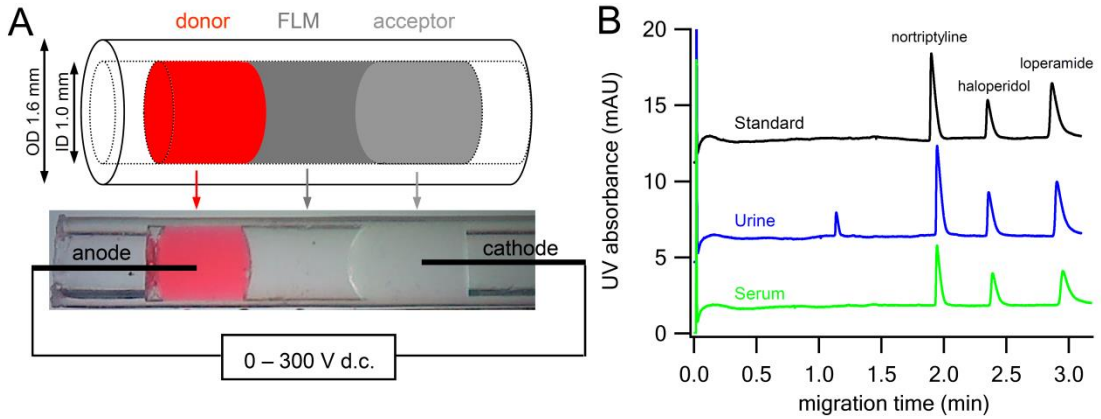

Fig. 1. A. Instrumental arrangement of μ-EME across FLM in PFA tubing (volume of each phase is 1.5 μL). B. μ-EMEs of three basic drugs from undiluted human body fluids followed by CE-UV of the acceptor solutions.

Off-line handling of acceptor solutions and their manual transfer to analytical instrument belong among the most frequently reported drawbacks of newly developed microextraction procedures. Alternative ways for direct combination of microextractions with analytical techniques are thus desirable and various approaches

for in-line coupling of EME and SLM/PIM extractions to commercial CE have been presented recently in our laboratory [6-8]. A simple, disposable microextraction device based on planar membranes (dialysis, SLM, PIM), which is compatible with injection systems of commercial CE instruments, is shown in Figure 2A. A fully automated extraction, injection, separation and quantification of target analytes in µL volumes of undiluted body fluids can be performed in a few minutes. Corresponding electropherograms for PIM extractions coupled in-line to CE analyses for determination of formate (the major metabolite in methanol poisoning) using this device are depicted in Figure 2B.

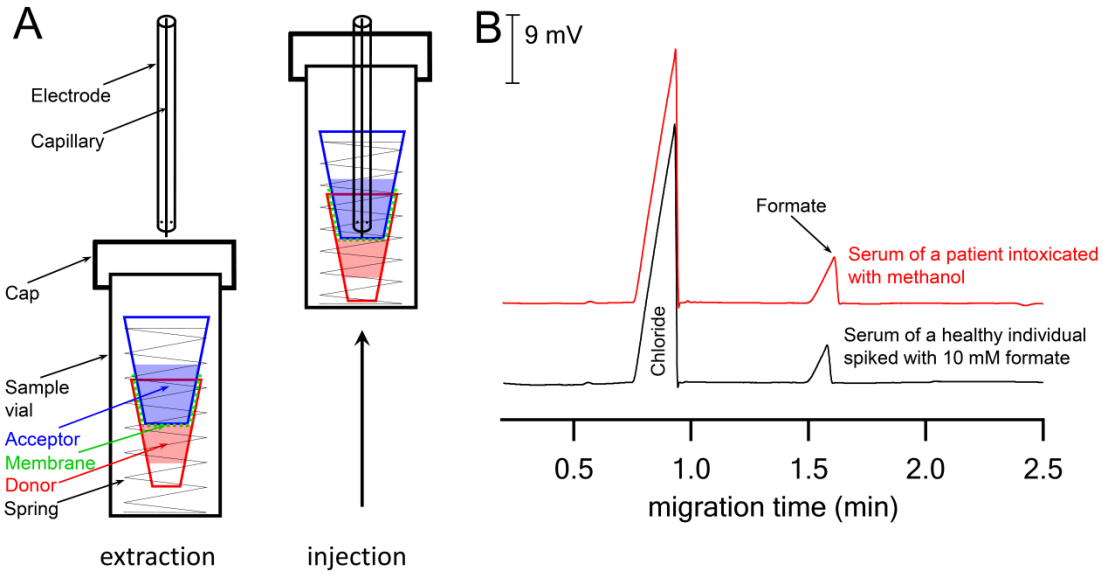

Fig. 2. A. Microextraction device with planar membrane in-line coupled to injection system of Agilent 7100 CE instrument. B. Direct analysis of formate in undiluted serum using PIM microextraction device in-line coupled to CE.

4 Conclusions

Novel LPME approaches for direct analyses of untreated biological samples were presented. They involved development and applications of new phase interfaces, such as FLMs and PIMs, down-scaling of EMEs to micro- and nano-format and development of disposable microextraction devices with planar membranes compatible with injection systems of home-made and commercial CE instruments. Particularly, in-line coupling of microextractions to commercial CE, which ensures full automation of the extraction, injection, separation and quantitation of target analytes, renders the developed techniques very attractive for routine clinical analyses.

Acknowledgement

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SEARCHING FOR GLYCAN CANCER BIOMARKERS: A COMBINED USE OF MASS-SPECTROMETRIC AND MICROCHIP CZE DATA

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Summary

Aberrant glycosylation for a number of proteins in physiological fluids has been observed in different human cancers. During most of the recent discoveries in cancer glycobiology, the moderm mass- spectrometric (MS) measurement technologies have played a decisive role in structural identification of the aberrant glycans and glycopeptides. Our recent studies using MALDI/MS-based glycomic profiling implicate various tri- and tetra-antennary N-glycans, in different quantitative ratios, to be indicative of several types of malignancy. Since certain fucosylated and multiply sialylated structures may occur as different isomeric glycan forms, MS alone is insufficient to distinguish isomerism. Capillary zone electrophoresis (CZE) with laser-induced fluorescence (LIF) of fluorescently labeled glycans can complement MS-based profiling with its capability to resolve isomeric oligosaccharides. The combined uses of MALDI/MS and microchip-based CZE on fairly extensive datasets from the ovarian cancer and colorectal cancer patients' samples could be statistically compared and quantified in our laboratories.

ELECTROCHEMISTRY OF BIOMACROMOLECULES AND ITS USE IN BIOMEDICINE

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Summary
In this review progress in electrochemistry of nucleic acids will be briefly summarized and new trends in electrochemical analysis of non-conjugated proteins and glycoproteins will be discussed particularly in relation to their application in biomedicine.

1 Introduction

First paper on electrochemistry of proteins was published in 1930 showing the ability of proteins to catalyze hydrogen evolution at Hg electrodes [1]. About 30 years later it was shown that DNA produced reduction signal reflecting DNA structure [2,3]. At present electrochemistry of nucleic acids (NAs) and proteins are booming fields (reviewed in [3,4]) NAs sensing deals predominantly with DNA hybridization and damage [3] with application in various areas in practical life, including biomedicine.

2 Experimental

Details of the experimental arrangements are given in the quoted literature [5-7].

3 Results and Discussion

In the recent decades electrochemistry of proteins was oriented mainly on a small group of conjugated proteins yielding reversible reactions of their non-protein components (e.g. metals in metalloproteins) while thousands of proteins important in proteomics, biomedicine, etc. were neglected. Recently we have shown that using constant current chronopotentiometric stripping (CPS) practically any protein produces electrocatalytic peak H at Hg and solid amalgam electrodes (SAEs). Using peak H at low current densities, proteins can be determined down to nM and subnanomolar concentrations. We have shown that proteins do not denature when adsorbed to Hg electrodes or SAE close to the potential of zero charge but can be denatured at negative potentials [4]. Enzymatic activity of urease attached to Hg electrodes was retained while prolonged exposure to negative potentials resulted in the enzyme denaturation. At higher current densities (where the rate of potential changes is extremely fast) CPS protein structure-sensitive analysis was developed [5,6]. At thiol-modified electrodes, changes in properties of mutant proteins could be detected [6]. Using CPS, detection of sequence-specific DNA-protein binding was possible [8].

In humans about 70% of proteins are glycosylated. Differences in protein glycosylation can be involved in pathological processes including cancer [9]. Combination of mass spectrometry with separation techniques has been successfully applied in studies of glycans and their glycosylation sites in glycoproteins [9,10]. For better understanding of progression of various forms of different diseases and for identification of new glycan-containing biomarkers for early diagnostics, simpler and less expensive methods are sought. For decades polysaccharides (PSs) were considered as electroinactive biopolymers. Recently it was shown that some PSs produce peak H_{PS} [7], similar to peak H of proteins. Very recently we have shown glycans containing N-acetylated glucosamine residues (which are electroinactive) can be easily deacetylated and transformed into electroactive species. Moreover, facile modification of PSs and oligosaccharides with osmium(VI) complexes transformed the electroinactive carbohydrates in electroactive Os(VI) adducts, detectable down to pM concentrations. Glycan detection in glycoproteins without deglycosylation was shown [11]. Our results show new possibilities in glycoprotein analysis [4].

4 Conclusions

Methods of electrochemical analysis are relatively simple and inexpensive. They can be easily miniaturized and adapted for parallel analysis. These methods can be applied not only for biomacromolecule determination but also for studies of their mutual interactions and of their properties at electrically charged surfaces.

Acknowledgement

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Abstracts of oral presentations – CECE Junior 2015

TILTED MICROPILLARS: A NEW ALTERNATIVE TO INCREASE MICROFLUIDIC CELL CAPTURE EFFICIENCY

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Summary
The design, fabrication and feasibility test of a novel microfluidic cell capture device is presented, exploiting a unique combination of proton beam writing and UV lithography to make lithographic irradiations under multiple target tilting angles and to easily reproduce large area structures, respectively. The microfabricated device is utilizing a novel doubly tilted micropillar array design for rare cell manipulation and capture from blood samples. Tilting the pillars increased their functional surface, therefore, enhanced the fluidic interaction with the special bioaffinity coatings, and improved fluid dynamic behavior as well. The proposed microstructures, designed by computational fluid dynamics simulations, were capable to support adequate distribution of body fluids and offered advanced cell capture capability on the functionalized surfaces. The hydrodynamic characteristics of the microfluidic systems were tested with yeast cells (similar size as red blood cells) for efficient capture of targeted cells.

1 Introduction

The limited availability of biological samples, mainly rare cells such as circulating tumor cells (CTCs) require improved microfluidic sample preparation devices and methods. Beyond the conventional microfluidic techniques a plethora of new designs have been developed; however, none of them utilizes tilted pillar arrays [1, 2]. In this presentation, we report on a novel combination of fabrication techniques of proton beam writing (PBW) [3] with soft lithography. PBW was applied to create highly precise tilted pillars, while the relatively large supporting parts (to accommodate

sample inlet and outlet) were fabricated by UV lithography. The unique novel combination of these different microfabrication methods represented an engineering challenge but resulted in a very effective tool, ideal for bioaffinity coating based microfluidic cell capture devices.

2 Experimental

Computational Fluid Dynamics (CFD) simulations were used to support the cell capture device design with a general goal of miniaturization to produce as compact systems as possible. COMSOL Multiphysics version 4.3.0.151 (Stockholm, Sweden) was the applied modelling environment. Then the effective cell capture part of the device was irradiated into PDMS using the scanning nuclear microprobe facility of MTA ATOMKI (Debrecen, Hungary) while the sample injection system was fabricated by UV lithography at the MEMS Lab of the Research Centre for Natural Sciences (Budapest, Hungary). Subsequently, the two parts of the cell capture device were precisely aligned using Süss Mikrotechnik MA6 (Garching, Germany) and bonded on a glass substrate by O_2 plasma treatment in a dedicated plasma chamber (TerraUniversal Plasma-Preen, Fullerton, CA, USA). Finally, the hydrodynamic characteristic of the microfluidic system was monitored by an upright microscope (Zeiss AxioScope A1, Jena, Germany) observing the movement of yeast cells (similar size as red blood cells).

3 Results and Discussion

This is the first study reporting on the fabrication of microfluidic cell capture devices using the unique combination of proton beam writing for lithographic irradiations under multiple target tilting angles followed by UV lithography to easily reproduce large area structures. CFD simulations revealed that pillar tilting does not only increase their functional surface area by 6.4% to result in enhanced interaction of the transporting medium with the immobilized bioaffinity layer, but also maintained the proper hydrodynamic characteristics of the system. The experimental data collected on the particle movement behavior well agreed with the CFD modeling results, clearly demonstrating the unique asymmetric pressure and shear force distribution near the tilted micropillars. The compatibility of the applied structural materials with MEMS/NEMS (micro/nano-electromechanical systems) technology was successfully demonstrated, even considering the integration requirements for future subsystem development.

Acknowledgement

This work was partly supported by the TÁMOP 4.2.2.A-11/1/KONV-2012-0036 Project, co-financed by the European Union and European Social Fund. Support from the Hungarian Scientific Research Fund OTKA (K108366), the Momentum grant #97101 (MTA-PE Translational Glycomics) and MedInProt fellowship of the Hungarian Academy of Sciences are also gratefully acknowledged.

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CAPILLARY ELECTROPHORESIS-MASS SPECTROMETRY: AN EFFICIENT TOOL FOR MIDDLE-UP CHARACTERIZATION OF MONOCLONAL ANTIBODIES AND ANTIBODY-DRUG CONJUGATES

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Summary
This oral presentation reports the detailed study on the use of coupled capillary electrophoresis-mass spectrometry for the heterogeneity analysis of IgG-type monoclonal antibodies and antibody-drug conjugates.

1 Introduction

Monoclonal antibodies (mAbs) and - more recently - antibody-drug conjugates (ADCs) have taken a prominent and increasingly important position in today's drug development and production. In the biopharmaceutical field there is a growing demand for mass spectrometry (MS)-based techniques providing compositional characterization of intact therapeutic proteins. Prior to MS analysis, efficient separation often is mandatory in order to allow accurate detection of protein modifications and minor sample components. Capillary electrophoresis (CE) has the intrinsic capacity to produce narrow peaks for proteins and shows good possibilities for the separation of closely related protein variants and isoforms [1,2].

2 Experimental

The experiments were performed on a PA 800plus capillary electrophoresis system (Beckman Coulter, Brea, CA, USA). Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) were coated with a triple-layer coating of Polybrene and dextran sulfate prior to use [3]. Background electrolytes consisted out of diluted acetic acid. CE-MS coupling was realized via electrospray ionization (ESI)

using co-axial sheath-liquid interface (Agilent Technologies, Waldbronn, Germany). Electrospray in positive ionization mode was used and mass spectrometric settings were optimized to allow optimal transmission and efficient declustering of intact proteins.

3 Results and Discussion

In order to facilitate efficient ESI of mAbs and ADCs, a middle-up approach was used by decomposing the IgG to their heavy and light chains (HC/LC) or F(ab')2 and Fc/2 fragments before CE-MS analysis (Figure 1A; page 3). A non-covalent positively-charged capillary coating was applied to prevent protein adsorption and achieve separation of the 25-100 kDa fragments using an acidic background electrolyte (Figure 1B). CE-MS of infliximab allowed detailed assessment of the glycosylation and C-terminal lysine variation of the HC and/or Fc/2 fragments (Figure 1C). Obtained glycoforms patterns on the HC and Fc/2 fragments are compared and discussed and the separation of glycoforms is indicated. CE-MS of trastuzumab-based ADCs yielded separation and relative abundance of the various conjugates disclosing their binding stoichiometries. Quantitative determination of the drug-antibody ratios (DARs) of the individual fragments as well as the overall ADC sample were achieved and compared to the reference LC-MS assay. Last, some recent developments in the use of neutrally-coated capillaries and high resolution mass spectrometers for mAb analysis are presented.

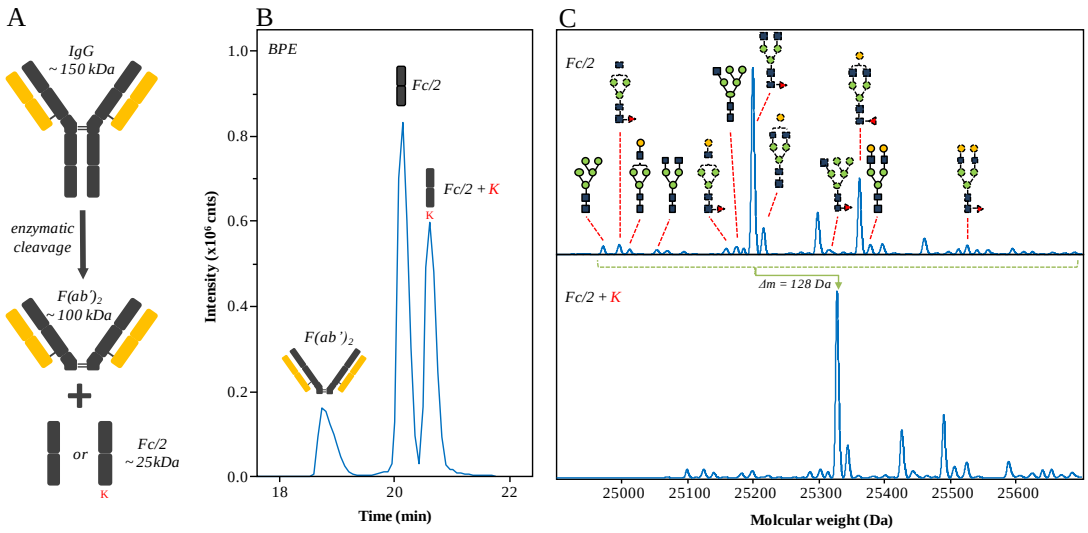

Fig. 1. Middle-up CE-MS characterization of infliximab. (A) Scheme of the fragments generated by enzymatic cleavage. (B) Base-peak electropherogram showing the separation of the fragments. (C) Mass spectra obtained in the apices of the two Fc/2 peaks illustrating the identified glycoforms and the mass shift caused by the addition of a lysine residue.

4 Conclusions

CE provides good separation conditions for mAb fragments and, moreover, the conditions allow their reliable MS detection. The developed CE-MS system enables the separation and detection of charge variants resulting from, for example, C-terminal lysine variants or drug conjugation. This allows for semi-quantitative and fragment-specific glycoform and conjugation profiles to be obtained in a single run.

Acknowledgement

Dr. Dennis Waalboer from the VU Medical Center is thanked for providing the trastuzumab-based antibody-drug conjugate.

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QUANTUM DOT-BASED IMMUNOPROBE FOR OPTICAL AND ELECTROCHEMICAL DETECTION

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Summary

Three-stage labeling strategy allows the preparation of specific conjugates composed of required antibodies and quantum dots as an extremely sensitive bio-probe applicable in varied immunoassays with following optical and electrochemical detection. This approach is essentially based on carbodiimide chemistry however each single step of whole procedure is separated and controlled which makes this applied

protocol oriented, highly efficient and it enables gain of pure antibody-conjugated quantum dots in ready-to-use condition.

1 Introduction

Quantum dots (QDs) have become popular and widely used fluorescent nanomaterial because of their excellent optical and electrochemical properties which are effects of material composition, inner structure and particle size. QDs dispose of sharp and narrow symmetrical emission spectra usually tunable according to their size, high quantum yield, high photostability and ideal water solubility which are important for compatibility with biorecognition molecules [1, 2].

Nowadays mostly one commercial conjugation kit (SiteClick™ Antibody Labeling Kits, Invitrogen, USA) based on antibody carbohydrate domain modification allowing the binding of maximally two QD particles on Fc fragment of antibody without final purification step and the commercial unavailability of secondary antibodies of required specificity labelled by QDs brought the idea to invent simple, highly efficient and reproducible protocol for preparation of these conjugates useful for variety of bioapplications.

2 Experimental

Antigen Apolipoprotein E (ApoE, BioVision Inc., Milpitas, California, USA) in amount related to monolayer was immobilized onto 1 mg of magnetic particles SiMAG-Carboxyl (1 μm, Chemicell GmbH, Berlin, Germany) by 2-step protocol through carbodiimide technique (EDC with sulfo-NHS in ratio of 6:1, Sigma-Aldrich, St. Louis, MO, US). Then whole aliquot of biofunctionalized particles was blocked by 0.1 M ethanolamine (Sigma-Aldrich, St. Louis, MO, USA) for 1 hour at RT. Blocking reagent was properly washed out and then the aliquot containing 0.5 mg of biofunctionalized particles was used for creation of immunocomplex with efficient amount of polyclonal anti-Apolipoprotein E antibodies (poly anti-ApoE; Moravian Biotechnology, Brno, Czech Republic) for 1.5 hours at RT. Thus prepared immunosorbent was activated for 10 minutes in presence of EDC with sulfo-NHS (6:1). After that solution containing EDC was discarded. For conjugation reaction the active volume was taken from 8 μM QDs stock solution (Qdot® 565 ITK™ carboxyl quantum dots made from CdSe/ZnS, Invitrogen, USA). Overnight binding of QDs at 4°C followed. At the end effective elution of labelled antibodies was realized by using 0.05% Trifluoroacetic acid (TFA) with 0.5% SDS.

3 Results and Discussion

This elegant method consists of three basic but crucial steps (Fig. 1). First step is initiated by specific biofunctionalization of magnetic carrier which is an essential element in this protocol. Antigen modified magnetic particles represent specific anchor enabling easier manipulation with target product at each level. Second step represents the creation of immunocomplex between required antibody and antigen covalently fixed on particle surface which ensures that active site of antibody is protected and then blocked for following conjugation with QDs. Due to this fact only

Fc fragment of antibody is exposed to efficient labeling reaction. Critical conjugation step is performed by using 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) as cross-linker supplemented by ester N-hydroxysulfosuccinimide (sulfo-NHS) for preactivation of functional groups of antibodies. Final elution of target antibody labelled by QDs is usually carried out by acidic medium with the addition of detergent.

TBE PAGE with UV detection and fluorescence spectra measurements were used as qualitative detection techniques (Fig. 2a, 2b). For evaluation yield of conjugation reaction anodic stripping voltammetry (square wave mode) was used (Fig. 2c).

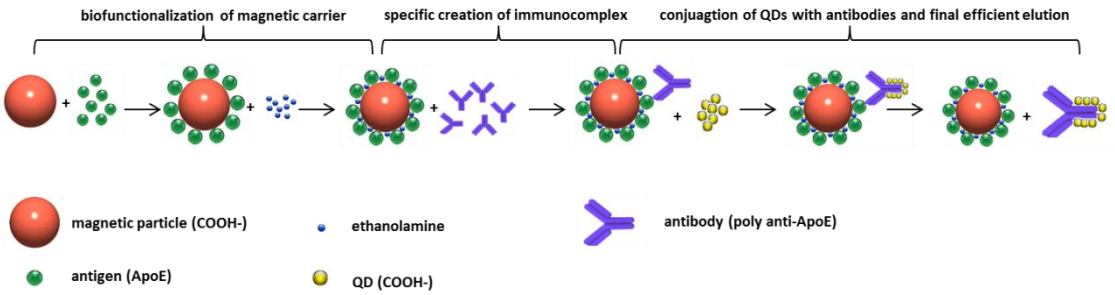

Fig. 1. Scheme of advanced conjugation procedure.

4 Conclusion

Practicability and effectiveness of our novel labeling procedure was tested and confirmed by chosen detection methods. Suggested protocol is expedient and it should be used as common technique for routine preparation of quantum dot-based conjugates of varied required specification especially for electrochemical and optical detection. This is kind of available protocol which can be applied in each laboratory with standard equipment. What is more this handy method provides only pure fraction of antibodies labelled by quantum dots which is really important and beneficial for further bioassays. Target analyte - antigen could be proved directly and quickly because of specific antibody and QDs as indicator visualize specific interaction between biomolecules.

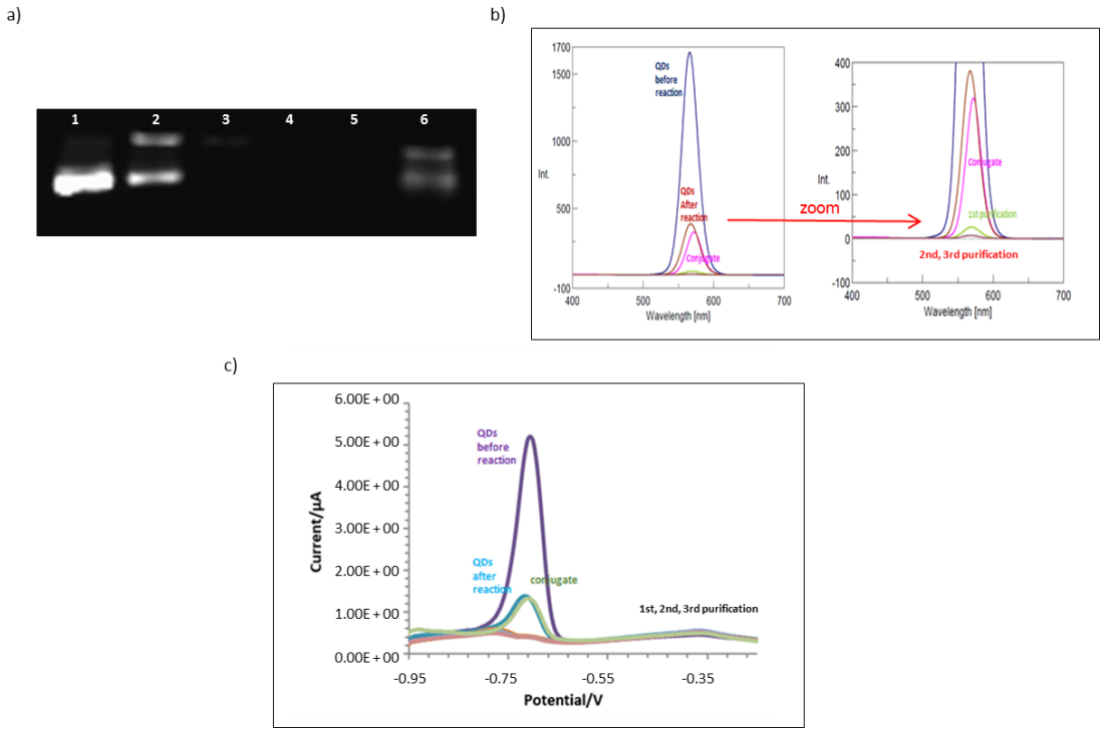

Fig. 2. Set of evaluation methods: a) TBE PAGE (1 – initial sample; 2 – binding fraction after conjugation reaction; 3,4,5 – washing fraction; 6 – conjugate; ChemiDoc™ XRS+ Imaging System with Image Lab™ Software); b) fluorescence spectra measurements (spectrofluorimetr Jasco FP-8500, SAF-851, one-drop analysis, 340 nm excitation); c) electrochemical detection - anodic stripping voltammetry (square wave) with screen printed carbon electrodes modified by mercury film.

Acknowledgement

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NANOSTRUCTURED GOLD ELECTRODES FOR DETERMINATION OF GLUCOSE IN BLOOD

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Summary
Fast and sensitive devices for monitoring of glucose are crucial in today's medicine. Most of the commercial glucometers are currently based on enzymatic catalysis which provides reasonable sensitivity but has a drawback in limited enzyme stability. With the development of novel surfaces, new generation of non-enzymatic sensors characterized by high sensitivity and stability is emerging. In this work, gelatin-templated nanostructured gold electrodes were developed and applied for non-enzymatic glucose determination. The analysis was done by direct electrochemical oxidation during cyclic voltammetry and amperometry with the limit of detection at 1.3 μM. The ability to detect physiological levels glucose in presence of interferents was demonstrated on deproteinized human blood serum.

1 Introduction

Electrodes with nanostructured surfaces provide a possibility to improve the sensitivity of electrochemical detection since they can accelerate electrode reactions with slow kinetics such as non-enzymatic electrooxidation of glucose [1]. The mechanisms of glucose conversion during cyclic voltammetry (CV) starts with adsorption of glucose on the electrode where it becomes oxidized by the catalysis of OH^- in the forward scan followed by the second oxidation in the backward scan which is catalyzed by O^{2-} formed during the reduction of gold oxide [2].

In recent years, different types of nanostructured electrodes have been developed to enhance electrochemical sensing [3, 4]. One possibility is to create nanostructures using the gel-based templating during which the gold created by reduction of gold(III) chloride is being deposited in the pores of the gel [5].

2 Experimental

2.1 Preparation of electrodes

Gold layer was sputtered on glass wafer with chromium adhesion layer and round electrode pattern was created using photolithographic technique. The electrode was then dipped into heated gelatin solution and the captured gel was crosslinked using glutaraldehyde. The nanostructured gold was deposited in electroplating bath containing gold(III) chloride hydrate under different conditions in order to select

electrode with optimal properties. Electrochemical characterization of the surfaces was done by CV on Autolab PGSTAT302N (Autolab, Netherlands). Surface visualization was performed using scanning electron microscope MIRA3 (Tescan, Czech Republic) and atomic force microscope Dimension FastScan (Bruker, Germany).

2.2 Glucose measurements in blood serum

Calibration serum LYONORM (Erba Lachema, Czech Republic) and real human sera from patients (kindly provided by Faculty of Medicine, Masaryk University, Czech Republic) were first deproteinized by acetone. The samples were centrifuged and pH was adjusted using 100 mM KOH [6]. Standard addition method was used to determine the glucose concentration in real samples. The results were verified by commercial glucometer ACCU-CHEK Active (Roche, Switzerland).

3 Results and Discussion

3.1 Characterization of electrodes

The morphology of created nanoscopic surfaces was dependent on current density during deposition, deposition time, gel thickness and hardening. Two main types of nanostructures were formed: the saw-like structures were created when using thick layers of gelatin or high degrees of hardening, while the volcanic stone-like structures were generated in the opposite conditions. Fig. 1 shows the AFM scan of electrode templated by gelatin hardened by vapors of glutaraldehyde.

Electrochemical properties of the electrodes were measured using CV in 0.5 M H_2SO_4 and in 1 mM $K_3[Fe(CN)_6]$ solution. Compared to the bare gold electrode, the electroactive surface area of gelatin templated nanostructures was magnified 60 times.

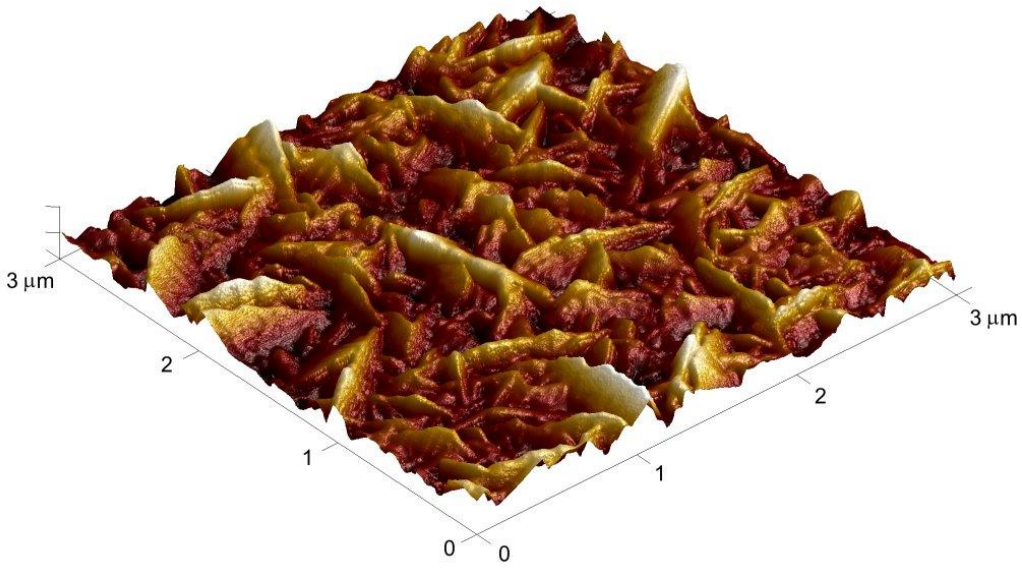

Fig. 1. AFM scan of gelatin-templated gold electrode. Plates with length of 1 µm and width around 50 nm were formed using gelatin template hardened by glutaraldehyde.

3.2 Detection of glucose

In order to evaluate sensor performance, CV of glucose solutions in 50 mM KOH was performed. A limit of detection (LOD) of 90 µM and linear range from 32 µM to 10 mM was achieved for CV while amperometry allowed to further improve the LOD to 1.3 µM.

The selectivity of this non-enzymatic approach was studied prior to detection of glucose in real samples. Typical interferents present in blood (ascorbate, dopamine, lactate and urea) were dissolved in KOH to 1 mM concentration and CV was measured. While oxidation was observed for all of these substances, the peaks did not overlap with peaks of glucose oxidation and therefore glucose detection was not hindered.

Human serum was deproteinized using acetone and pH was adjusted by 100 mM KOH. CV of alkalized serum was measured followed by additions of glucose (Fig. 2). The original concentration in serum was then evaluated by standard addition method. Relative standard deviation of 4 % and excellent correlation with glucometer results were achieved. This demonstrates the potential of the developed nanostructured sensor for the use in clinical analyses.

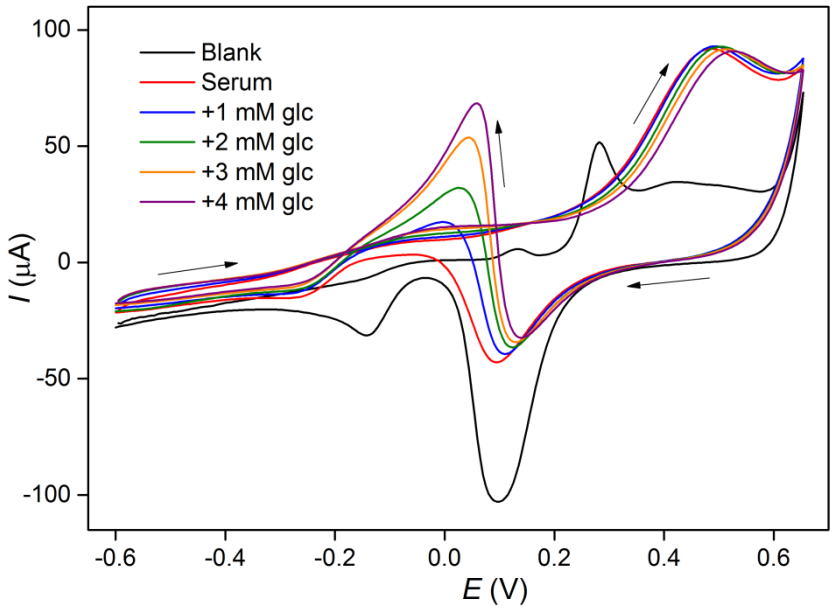

Fig. 2. Cyclic voltammetry of deproteinized human serum with four additions of standard glucose solution.

4 Conclusions

Gelatin-templated gold nanostructured electrodes were developed and used for non-enzymatic determination of glucose. Compared to bare gold, 60 fold increase of electroactive surface area was achieved. Glucose was initially analyzed in KOH to optimize the assay parameters and to study the process of electrooxidation. Limit of detection of 1.3 µM and linear range up to 10 mM was achieved using amperometric

set-up. The detection in real serum samples was done after deproteinization by acetone and alkalization by KOH. Good correlation with results obtained using commercial glucometer and negligible interferences from other oxidizable substances from blood were observed. This demonstrate the potential of the sensor to be used in routine clinical analyses.

Acknowledgement
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NOVEL PEMPDA β–CYCLODEXTRIN STATIONARY PHASE, STUDY OF ITS SEPARATION POTENTIAL

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Summary
New HPLC column was prepared by dynamic coating of the ion-exchange stationary phase Luna 5µ SCX using the newly synthesized PEMPDA–β–CD chiral selector. The separation potential of this column was measured and compared with a commercially available β-CD column.

1 Introduction

New cyclodextrin (CD) derivatives are synthesized continuously. The derivatives often suffer from polydispersity and low yields [1–3]. New monosubstituted tetraalkylammonium derivative of β–CD (PEMPDA–β–CD) was prepared as a promising CD derivative (see Fig.1 for the structure) for application in separation

science. The complete synthesis of PEMPDA–β–CD was described and published by *Popr et al.* [3]. Two permanent positive charges of PEMPDA–β–CD are useful for preparation of the stationary phase (SP). In our work we focused on preparing and characterization of the new β–CD–based SP by dynamic coating. Achiral and chiral separations were evaluated for description of separation potential of the novel SP. The same separations were performed with commercial β–CD SP for comparison.

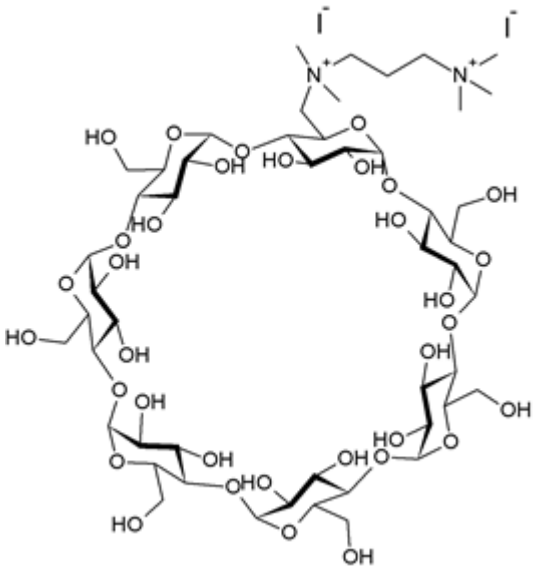

Fig. 1. Structure of PEMPDA-β-CD.

2 Experimental

2.1 HPLC equipment and columns

The Waters Alliance system (Waters Chromatography, Milford, MA, USA) was used. Luna 5µ SCX 100 Å, 250 x 4.6 mm, an ion exchange SP with benzenesulfonic acid on silica support (Phenomenex, Torrace, USA) and Cyclobond^{TM} I 2000 – β–CD column, 250 x 4.6 mm, (Astec, Whippany, USA), were used for dynamic coating with PEMPDA–β–CD and for comparative measurements, respectively.

2.2 Dynamic coating

PEMPDA–β–CD dissolved in aqueous solution of formic acid, pH 2.20, (c = 1 mg mL^{-1}) was coated on Luna 5µ SCX column at flow rate 1 mL min^{-1} for 1 hour.

2.3 Separation conditions

Mobile phases (MPs) used were composed of methanol (MeOH) and 10 mM ammonium acetate buffer (AMAC buffer), pH 4.00 in various volume ratios. Column temperature and sample temperature were set at 25°C, flow rate used was 1 mL min^{-1} and injected volume of the samples was 10 µL. UV detector with wavelength set to 254 nm was used for detection.

2.4 Analytes

Seven sets of achiral analytes were measured, *i.e.* benzene and polycyclic aromatic hydrocarbons; *n*-alkyl derivatives of benzene; aniline and its alkyl derivatives; positional isomers of xylene and cresol; pyrocatechol and resorcinol; positional isomers of dimethylphenol and five structurally different chiral analytes, *i.e.* three atropisomers, oxazepam and lorazepam.

3 Results and Discussion

Part of the chromatographic results obtained by our measurements is summarized in Table 1. One of the positive features of the separation system with PEMPDA–β–CD coated SP for separation of some achiral analytes are substantially shorter retention times than those obtained with the commercial β–CD column (see Table 1). If aniline and its derivatives were tested, different chromatographic behavior was observed. The high retention of these analytes on PEMPDA–β–CD column can be explained by interactions of their free NH_2 group/s with free benzenesulfonate groups that remain uncoated on SP surface. On the other hand, very low retention of aniline and its derivatives was observed in the system with commercial β–CD SP. In case of chiral analytes, very short retention times were obtained, even in MPs with low MeOH content, on the PEMPDA–β–CD coated chiral stationary phase (CSP), while high retention times were observed on the commercial CSP, even at higher MeOH content in the MP. Baseline separation was achieved only for oxazepam and partial separation for one of the atropisomers (see Table 1).

Table 1. Some chromatographic data k and R_S of achiral sets of analytes on coated and bonded CD-based SPs at given MP composition

Analyte	PEMPDA–β–CD coated SP			Analyte	Commercial β–CD SP		
	MP *(v/v)*	k	R_s		MP *(v/v)*	k	R_s
Toluene	30/70	1.39		Toluene	40/60	3.71	
Propylbenzene		4.79	8.69	Propylbenzene		12.38	10.93
Butylbenzene		8.73	5.25	Butylbenzene		20.53	3.91
Pentylbenzene		11.12	2.04	Pentylbenzene		27.50	1.91
Aniline	60/40	3.01		*N*,*N*–Diethylaniline	30/70	0.21	
N–Methylaniline		3.84	4.06	*N*–Ethylaniline		0.76	9.43
N–Ethylaniline		7.68	14.75	Aniline		0.92	3.25
N,*N*–Diethylaniline		31.39	33.08	*N*–Methylaniline		1.03	1.42
Pyrochatechol	20/80	0.67		Pyrocatechol	20/80	1.86	
Resorcinol		0.93	3.86	Resorcinol		2.20	2.20
Oxazepam	20/80	1.68	1.57	Oxazepam	30/70	1.60	2.96
Cl–HDDP	10/90	0.18	1.25	Cl–HDDP	40/60	54.72	1.25

MP composition: MeOH/10 mM AMAC buffer, pH 4.00 *(v/v)*, k – retention factor, k_1 – retention factor of the first eluted enantiomer, R_S – resolution.

4 Conclusions

In general, for shorter, less expensive and „greener“ analyzes of the sets of achiral compounds without basic group/s, the PEMPDA–β–CD coated SP can be the column of the first choice. In addition, a big advantage of the dynamically coated column is a possibility to simply exchange selector deposited on the stationary surface for a more suitable one and so, quickly and efficiently prepare a new type of SP.

Acknowledgement

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STRUCTURAL ANALYSIS AND RELATIVE QUANTIFICATION OF TIGHT JUNCTION PROTEINS: CLAUDIN-1 IN HUMAN SKIN BIOPSY USING CONFOCAL MICROSCOPE

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Summary

Epidermal tight junctions (TJ) represents crucial regulatory element of epidermal homeostasis. Since the skin creates barrier to external environment, epidermal TJs are exposed to various factors that may deregulate them. Deregulation may occur not only as altered quantity of TJ proteins but also as altered localization pattern. Therefore, human skin samples of young and aged individuals were analyzed by confocal laser scanning microscopy (CLSM). CLSM has enabled us to detect changes in localization of one of the main TJ protein, claudin-1 in photoaged skin sample.

1 Introduction

TJs are type of cellular junctions that are present mainly in epithelial tissues where they connect adjacent cells together sealing the paracellular space. Importance of TJs has been also discovered for the epidermis where they fulfill key roles in keeping epidermal homeostasis. Epidermal TJs contribute to calcium gradient, regulate biosynthesis and vesicular transport and create selective paracellular barrier [1]. Approximately 30 proteins that are divided into 3 groups: transmembrane, scaffolding and polarity proteins contributes to formation of functional TJ site in human skin and each of TJ related proteins have its specific expression pattern in the epidermis [2]. Various internal or external factors such as bacterial toxins, inflammation, pollutants or UV-light damage individual TJ proteins in the epidermis which can end up with aberrant formation of the stratum corneum and deteriorated barrier function of the skin [3]. Damage caused to TJ alters quantity as well as localization of TJ protein expression [4]. CLSM allows precise study of TJ protein expression patterns in any section and depth of the sample even for low expressed proteins. CLSM also offer the 3D imaging of the sample, which provides additional valuable information about protein expression. With use of hybrid detector (HyD), CLSM quantification is step further than ordinary photomultiplier.

2 Experimental

2.1 Immunohistochemistry and CLSM

Biopsies of human skin were obtained, mounted in freezing medium and stored under – 80 °C within less than hour after facial surgery. Cryostat sections (8-10 μm) of human skin samples were put on coated glass slides and fixed in precooled (-20 °C) acetone. After washing in TBS buffer IHC staining was performed. After blocking with 5 % BSA, mouse anti-claudin-1 antibody (clone 2H10D10, Novex) was incubated for 120 minutes. Samples were subsequently washed for 3x10 minutes. Afterwards, anti-mouse Alexa 555 coupled antibody was applied for 60 minutes at room temperature. Cell nuclei were stained with Hoechst 33342. Samples were then washed and mounted in anti-fade medium. Confocal microscope Leica SP8 was used for sample observation. HyD detector and constant performance settings were applied to all samples. Images has been evaluated by Leica software.

2.2 Western-blotting

Proteins were extracted from human skin samples using lysis buffer with protease and phosphatase inhibitor cocktail. Equal amounts of total protein (17 μg) were separated using SDS-PAGE and transferred to PVDF membrane. After blocking for 1 hour with 5 % skim milk specific primary antibodies (claudin-1) were applied overnight at room temperature. After washing step appropriate secondary antibodies coupled with horseradish peroxidase were applied for 30 minutes at room temperature. Immunoreaction was visualized by addition of substrate and chemiluminescence analysed by densitometer (UVITEC).

2.3 Microarray analysis

Keratinocytes were obtained by tape-stripping. The isolated mRNA was amplified, fluorescently labelled and hybridized to microarray slides (Human Gene Expression 4x44K v2 chip, Agilent). The data were normalized and differential gene expression was determined between the two age groups.

3 Results and discussion

CLSM has been proved to be a powerful tool for structural analysis and reliable for relative quantification of TJ proteins, claudin-1. Microarray analysis showed altered expression of claudin-1 on mRNA level. Aged epidermis exhibited significantly decreased expression of claudin-1 mRNA. Although claudin-1 expression on protein level had similar pattern, the difference was not as significant as on mRNA level. Analysis of claudin-1 by western-blotting confirmed these results. However, structural study and 3D imaging by CLSM revealed structural changes of claudin-1 expression that would be hardly discoverable otherwise. Claudin-1 was more concentrated at cell membranes in the epidermis of young subjects and mainly in stratum granulosum while aged epidermis exhibited rather scattered expression pattern of claudin-1. These results suggests that one of the main TJ proteins, claudin-1 has different expression pattern in aged skin in comparison to young. Lowered expression of claudin-1 in aged epidermis was observed also in mice [4]. Question is whether deregulation of claudin-1 is caused directly by UV exposure or by other factors. Since localization of claudin-1 is also impaired scaffolding or polarity proteins are probably deregulated as well [5]. Moreover, we also observed slightly thicker stratum corneum in aged epidermis using transmission detector.

4 Conclusion

Although relative quantification of claudin-1 protein by CLSM did not find difference as significant as on mRNA level, impaired localization of claudin-1 was observed in aged epidermis. Therefore, analysis of TJ protein localization in this regard is indispensable and CLSM proved to be a valuable tool. However, further studies are needed to find the nature of claudin-1 deregulation.

Acknowledgements

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COMPARISON OF MODELS USED FOR DESCRIPTION AND PREDICTION OF RETENTION BEHAVIOR OF OLIGOSACCHARIDES IN HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY

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Summary
In this work, retention of selected maltooligosaccharides in hydrophilic interaction liquid chromatography was investigated. In gradient elution mode, the increased peak capacities are achieved with respect to the isocratic elution, which allows better separation of complex mixtures. For optimization of gradient conditions it is necessary to accurately describe the retention in dependency on mobile phase composition. Thus we have evaluated different retention models and applied them for prediction of gradient retention factors of fluorescently labelled oligosaccharides.

1 Introduction

Exact description of retention of compounds in liquid chromatography is necessary for optimization of separation with programmed change of elution conditions. Several models were proposed for fundamental description of retention in reversed-phase liquid chromatography and hydrophilic interaction liquid chromatography (HILIC) [1-6]. When a wide range of concentration of an organic modifier is used, the retention can exhibit non-linear behavior due to the interactions between solutes and solvents, which are not considered in simple linear models. The addition of ionic compounds into mobile phases also significantly affects the retention in HILIC if sufficient ionic strength is maintained [7]. In this work, we have evaluated suitability of different models for prediction and optimization of gradient conditions in HILIC using fluorescently labelled oligosaccharides. Based on isocratic retention data, we have predicted gradient retention factors and compared them with experimentally determined ones. Finally, we have evaluated the influence of separation conditions on peak capacities achievable in HILIC separation of native and fluorescently labelled maltooligosaccharides.

2 Experimental

HILIC separation of maltooligosaccharides (maltose to maltoheptaose) was conducted using Shimadzu modular liquid chromatograph consisted of two LC-20ADXR pumps

connected with 20 μL mixer in high-pressure gradient system and SIL-20ADXR autosampler (all Shimadzu, Kyoto, Japan). The following columns were used: Ascentis Silica-gel, TSK gel Amide-80, Luna NH2, GlycanPac AXH-1 and ZIC-HILIC columns (all 1 mm i.d.). Detection was performed using the ESI-MS in both, positive and negative ion mode. Isocratic and gradient separations were performed at 30 °C in acetonitrile/water mobile phases with addition of acetic acid and ammonium acetate. Maltooligosaccharides were separated in native form and derivatized by 2-aminobenzoic acid (2-AA), 2-aminobenzamide (2-AB) and 8-aminonaphtalene-1,3,6-trisulfonic acid (ANTS).

3 Results and Discussion

The retention of native and fluorescently labelled maltooligosaccharides was studied in HILIC using various stationary phases including silica gel, aminopropyl silica gel, amide (carbamoyl bonded silica), glycan pack, and ZIC-HILIC zwitterionic phase. The partitioning of the analytes between the bulk mobile phase and adsorbed water-rich layer, polar and ionic interactions of analytes with stationary phase have been evaluated and compared. While native oligosaccharides are well separated on most of the stationary phases used, the anionic ANTS and 2-AA derivatives are weakly retained on bare silica and amide phases. The effects of the mobile phase additives on retention were described. Addition of ammonium acetate yielded stronger retention of both native and labelled oligosaccharides due to the higher ionic strength of mobile phase in comparison with addition of acetic acid. The suitability of different models for prediction of retention including linear, quadratic, mixed-mode and empirical model was tested. Best description of retention was achieved using quadratic and mixed-mode model accounting for solute-solvent interactions and thus non-linear behavior. Furthermore, the conditions for fast gradient separation of oligosaccharides were predicted using isocratic data and the peak capacities for all separation conditions were compared.

4 Conclusions

Retention of native and derivatized maltooligosaccharides at five different stationary phases was described by using four models. The mixed-mode model was the most suitable, taking into account solutes partitioning in rich-water layer, for solvent - solutes and solvent – solute – stationary phase interactions. The model was applied for prediction of gradient retention factors and the results were compared with the experimentally achieved gradient data.

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INFORMATION ENTROPY CALCULATION TECHNIQUE FOR THE DETECTION OF THE PHASES IN THE SELF-ORGANIZING REACTION

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Summary
We are searching for an analytical tool for the detection of the self-organizing system's states, which are classified on the basis of the organization of the system's compounds. Based on the Information Theory Postulates, we derived a point information gain and point information gain entropy density as metrics of information space and unique characteristics of event of interest in the system during examined time interval. The developed method was verified on the detection of the phases of the Belousov-Zhabotinsky reaction. Using multivariate analysis, the point information gain entropy classified the trajectory of the structures' formation into consecutive clusters. This proved that the point information gain may be used for classification of timely stable structures of the system's trajectory. It proposes the information yield analysis approach for the statistical meta-modeling and prediction of the complex systems' behavior.

1 Introduction

Created more than half century ago [1], Belousov-Zhabotinsky (BZ) reaction does not cease to inspire the scientists nowadays. Its reaction mechanism contains more than 80 reaction equations with more than 26 different components [2], and became the brightest example of the non-equilibrium thermodynamics [3]. A distinguished feature of the BZ reaction is a diversity of the space patterns (chemical waves) visible by

naked eye [4]. These patterns change when the reaction is performed in a (ca. 3-mm) thin layer in a Petri dish. The BZ system imitates the behavior of the self-organizing systems such as organisms with a higher level of complexity [5]. Thus, the techniques used for the analysis of the BZ reaction could be potentially applied to a wide range of other systems (populations of individuals or living cell colonies) which seem to be described by the same mathematical model.

In this contribution, we verify an algorithm, which connects the approaches of information entropy and multivariate clustering, on the detection of phases of the self-organizing BZ reaction.

2 Experimental

For experiments, the recipe for oscillating bromate-ferroin-bromomalonic acid reaction modified by Dr. Jack Cohen [6, 7] was chosen. Experiments were performed in a standard Petri dish on a temperature controlled steel desk at 27°C. The chemical waves were recorded by a Nikon D90 camera in the regime of the Time Lapse Shooting (1 snapshot/10 s). The images were processed by the algorithm [8, 9], which is based on the calculation of the Rényi information weight [10] of each pixel in the complex system of the geometrical structures (waves). This measure is called a point information gain [bit]

$$\gamma_\alpha(x,y) = \frac{1}{1-\alpha}\log_2\left(\sum_{i=1}^{n} p_{i,x,y}^{\alpha}\right) - \frac{1}{1-\alpha}\log_2\left(\sum_{i=1}^{n} p_i^{\alpha}\right),$$

where p_i and $p_{i,x,y}$ are probabilities of occurrences of the n intensities (i) with and without the examined event (the pixel at the coordinates (x,y) in the R, G, or B image channel), respectively. By changing the order of the entropy α = {0.1, 0.3, 0.5, 0.7, 0.99, 1.3, 1.5, 1.7, 2.0, 2.5, 3.0, 3.5, 4.0}, we focused on the events with different probabilities of occurrences in the intensity distribution. The cumulative variable point information gain entropy density [bit] was consequently calculated as a sum of all $\gamma_\alpha(x, y)$ for k occupied intensity levels at given α, i.e.,

$$\Xi_\alpha = \sum_{i=1}^{k} \gamma_\alpha(x,y).$$

The last step of the calculation was the separation of the state trajectory of the BZ reaction into individual contributions via cluster analysis of $\Xi_\alpha(\alpha)$-spectra (k-means clustering with Squared Euclidian distance). The state trajectory was depicted as color separated scores in the space of the first 3 principal components [11].

3 Results and Discussion

The waves' formation in the BZ reaction occurs due to the oxidation-reduction cascade with the participation of the ferroin complex as a reaction catalyst. The ratio of $Fe(phen)_3^{2+}/Fe(phen)_3^{3+}$ is directly as well as indirectly connected to the products/reagents balance in the system. The spectral properties of the ferroin visualize the reaction process [12, 13] via tracing of its dynamical concentration changes in the R, G, and B image intensity profiles (Fig. 1). In other words, the pixel

intensity contains the information about the chemical kinetics of the whole scope of reactions in the BZ system which leads to the oscillation dynamics. This whole scope of interactions was analyzed by information entropy. Since the Rényi entropy (I_α) [10] is connected to the generalized dimension (D_α) through the relation $D_\alpha = \lim_{r\to 0} \frac{I_\alpha}{\log r}$, where r is the size of the box (pixel), the $\gamma_\alpha(x,y)$ spectrum reflects the space structure of the examined multifractal object. Consequently, the time evolution of the $\gamma_\alpha(x,y)$ examines the complete reaction kinetics and reagents/products concentration interrelation and, thus, an overall complexity of the process. For infinite number of α, Ξ_α create a state space in which the point is unique for each pair of images with different spectral properties. Ξ_α-spectra are unique properties of the system's states, and, using the multivariate analysis, group the cascade of the successive system's evolutions into phases (e.g., Fig. 2).

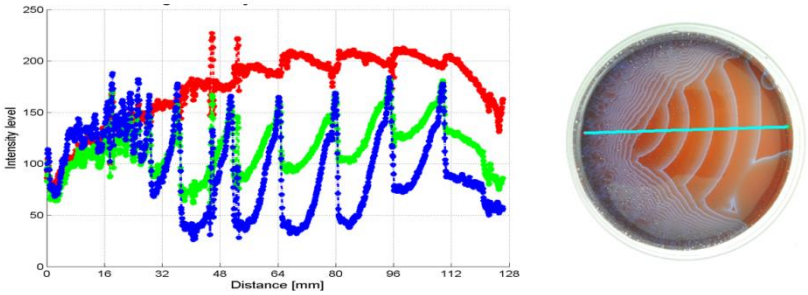

Fig. 1. Intensity profiles of the wave dynamics in a step of the Belousov-Zhabotinsky reaction, which trace the $Fe(phen)_3^{2+}/Fe(phen)_3^{3+}$ concentration changes. Colors of the curves correspond to the image (R, G, B) channels.

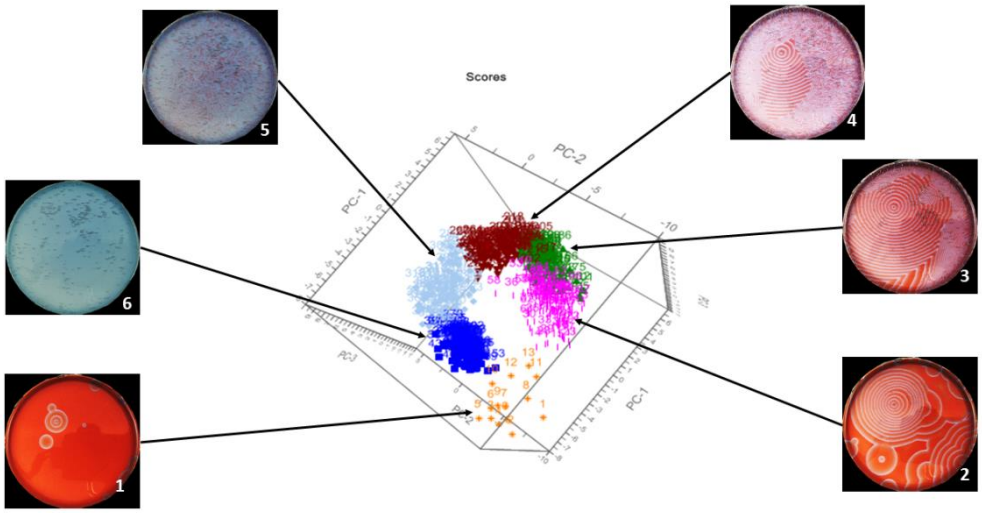

Fig. 2. Phases of the Belousov-Zhabotinsky reaction detected via information entropy. Visualized in the space of 3 principal components, where the color separated data (clusters) shows the dispersion of the system's evaluation over time.

4 Conclusions

Our approach for the detection of the phases in the self-organizing chemical reaction combines the information about the chemical processes in the system over time with the spatial and structural organization of active reaction centers. Cluster analysis of $\Xi_\alpha(\alpha)$-spectra divides the image sequence into groups identical to the states of the system trajectory. The verification of the algorithm on the simple model of self-organization offers a promising direction for the automated analysis of the complex systems' dynamics.

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PIECES OF KNOWLEDGE FROM THE STUDY ON THE ELECTROPHORETIC BEHAVIOUR OF SHORT OLIGODEOXYRIBONUCLEOTIDES IN FUSED SILICA CAPILLARIES

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Summary
The presented study was focused on the investigation of the influence of background electrolyte (BGE) pH, composition and concentration on the electrophoretic transport of short synthetic oligodeoxyribonucleotides (ODNs) in fused silica capillaries. Both mobilities and separation efficiencies of the ODNs were affected by variations in the background electrolyte characteristics. However, for a systematic explanation of the observed variations in ODNs characteristics, the changes in the BGE properties and the acid-base properties of ODNs are not sufficient. There are other relevant processes, participating in capillary electrophoretic separations of short ODNs, and these will be discussed.

1 Introduction

Oligonucleotides and their derivatives are frequently used tools in e. g. biological and biochemical studies of DNA [1, 2]. Short ODNs, which consist of maximum 10 nucleotide units, are classified as molecules of small or intermediate size. Therefore their mixtures can be separated by capillary electrophoresis (CE) in buffered aqueous solutions. However, detailed studies of short ODNs (and of their derivatives) are scarce. Available literature dealing with ODNs separations does not make it possible to create any general idea of the electrophoretic behaviour of ODNs [3, 4]. Majority of published separations use pH above 8.5, at which ODNs migrate anionically because they bear excess negative charge [5]. This is probably the fact behind the generally accepted opinion that the electrophoretic behaviour of ODNs is wholly determined by their negative charge. Articles dealing with ODNs properties different from the acid-base ones are exceptions in fact [6, 7].

2 Experimental

CE experiments were performed using a laboratory set-up based on JASCO 875 UV-VIS spectrophotometric detector (Jasco, Tokyo, Japan), and high-voltage power supply Spellman 1000E (Plainview, New York, USA). Separated zones were detected at 260 nm. Detector output was monitored by Clarity software in CE modification (DataApex, Prague, Czech Republic). For the elimination of electroosmotic flow which causes experimental difficulties, the inner capillary wall was coated with chemically bound polyacrylamide. The coated capillary was used in experiments in the pH range 4.7 – 7.6. In experiments performed at higher pH uncoated capillary was

used. Synthetic ODNs have been supplied by VBC Biotech Services GmbH (Vienna, Austria) and GENERI Biotech (Hradec Králové, Czech Republic).

3 Results and Discussion

The starting motivation of this study was the general opinion, that electrophoretic properties of ODNs are determined by their negative charge and that anionically migrating ODNs are indifferent to the negatively charged fused silica capillary surface [3].

In the first experiments, an ODN consisting of four nucleotides with adenine and one nucleotide with thymine (sequence AAAAT), was not detected from first injection into the capillary filled with 20 mM sodium acetate buffer pH 4.7. Only two zones of AAAAT were detected from three subsequent analyses. However, when the acetate buffer pH was adjusted with TRIS or morpholine, the ODN migrated reproducibly.

Thus next experiments have been an attempt to reveal the action of organic cations in ODNs transport through fused silica capillary. Monovalent and divalent cations as additives in sodium acetate buffer provided for a reproducible migration of ODNs, however the separation efficiency was lowered. Trivalent and tetravalent organic cations resulted in an unacceptable decrease in ODNs mobilities. Only the higher concentration (5 mM) of mono- and divalent cations added into morpholine acetate buffer yielded separation efficiency improvement, however at the cost of decreased mobility.

The continuation of experiments in polyacrylamide coated capillary comprised the use of zwitterionic Good´s buffers MES (pK_a = 6.4 [8]) and MOPS (pK_a 7.2 [8]). ODN AAAAT was not detected when injected in long intervals in capillary filled with 20 mM sodium-MES buffer. However if morpholine was the BGE cation, the ODN was detected and its migration was reproducible. In 20 mM sodium-MOPS buffer reproducible migration of ODNs was reached for the first time for NaOH-adjusted buffers. When the buffer concentration was 40 mM, the separation efficiency of AAAAT peak rose unexpectably, up to $3.5 \cdot 10^5$ theoretical plates.

In experiments with alkaline BGEs, uncoated capillary was used. From BGEs most widely used for analyses of nucleotides and their oligomers - borate, phosphate, carbonate - only carbonate of pH 9.5 was chosen. The influence of carbonate buffer concentration on ODNs migration was also checked – and revealed permanent decrease in mobilities and increase in separation efficiencies of ODNs. If ethanolamine (pKa 9.5 [8]) was the buffering BGE constituent, dependency of ODNs mobilities on the BGE anion was proven.

In summary, our experiments show that mobilities of ODNs depend on the buffer cation, buffer anion, buffer concentration, analyte concentration and analyte identity. Separation efficiencies depend on all the above mentioned variables too.

4 Conclusions

The experimental data show that the electrophoretic properties of ODNs are not as simple as they have been presented in the available literature until now. In spite of the

complex ODNs behaviour, we show that very high separation efficiencies of ODNs peaks are attainable. The conference contribution will discuss possible processes that are relevant for CE separations of short ODNs.

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LABEL FREE PROTEIN ANALYSIS AT CARBON ELECTRODES

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Summary
We show that electro-oxidation of tyrosine (Tyr), tryptophan (Trp) and histidine (His) residues at carbon electrodes can be used for structure-sensitive analysis of a large number of proteins, including those important in biomedicine, such as oncoprotein Anterior Gradient 2 (AGR2).

1 Introduction
Considering the present fast development in proteomics and applications of electrochemical methods in genomics and biomedicine, we are currently developing electrochemical methods for protein [1,2] and protein-nucleic acid analysis [3].

Electro-oxidation of Tyr and Trp residues in proteins and peptides at positive potentials far from zero was found 35 years ago [1]. Recently we showed that

oxidation peak of Tyr, Trp residues can be used for discrimination between native and denatured forms of human serum albumin and of other proteins [4].

In this work we tested possibilities of utilization of carbon electrode in label free protein analysis.

2 Experimental

The experiments were performed with a three electrode system connected to a μAutolab III potentiostat (Metrohm-Autolab). The working electrode was a glassy carbon electrode (GCE, 3.14 mm^2), a basal plane pyrolytic graphite electrode (BPGE; 7.5 mm^2), a edge plane pyrolytic graphite electrode (EPGE; 7.1 mm^2), a screen-printed carbon electrode (SPCE, 12.6 mm^2; Dropsens, Spain) and a carbon paste electrode (CPE, 7.65 mm^2) controlled by a VA-stand 663 (Metrohm). Ag|AgCl|3 M KCl was used as the reference electrode and platinum wire as the counter electrode.

3 Results and Discussion

We demonstrated that various carbon electrodes, including CPE, GCE, EPGE and also SPCE in connection with adsorptive stripping SWV, are applicable for detecting conformational changes of proteins due to their denaturation. The EPGE, as compared to the other forms of carbon electrodes, seems to be the most suitable carbon material for investigating conformational changes in proteins [4]. In addition, His-tagged and non-tagged forms of proteins such as AGR2, Glutathione-S-transferase, α-synuclein and cytochrome b5 were studied [5]. In addition to oxidation peak of Tyr and Trp, His-tagged forms yielded characteristic electro-oxidation peak of histidine. In absence of the His-tag in His-containing proteins, appearance of histidine peak was influenced by accessibility of His residues, and depended on the carbon electrode type.

4 Conclusions

Oxidation histidine peak, in combination with Tyr and Trp oxidation responses may find use in label-free analysis of numerous proteins, including those important in biomedicine.

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NOVEL POLYELCTROLYTES USED AS PHYSICALLY ADSORBED COATINGS – CAPILLARY ELECTROPHORESIS AND QUARTZ CRYSTAL MICROBALANCE STUDY

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Summary
Ionic liquids are defined as salts molten at temperatures below 100 °C. Polymeric ionic liquids (PILs), on top of that, are class of polyelectrolytes (PECs) made by polymerization of monomers of ionic liquids, where a positively/negatively charged polymer is countered with a monomeric organic/inorganic ion. In this work we present two newly synthesized PILs utilized for physically adsorbed cationic coating of fused silica capillary. Coating stability and EOF were studied in pH range 3 - 10, with different buffers, and ionic strengths. Coating adsorption dynamics along with PIL layer thickness was analyzed using quartz crystal microbalance with dissipation monitoring (QCM-D). Set of alkyl benzoates was used for determination of coating hydrophobicity. Utility of the system was further evaluated with capillary electrophoresis (CE) of commonly abused drugs in doping - set of beta-blockers. Finally, a method for analysis of 1,5-diazabicyclo[4.3.0]non-5-ene acetate ([DBNH][OAc]) products in low pH was developed.

1 Introduction

Fused silica capillaries are the most commonly used type of capillary for CE analysis. Here, EOF is based on ionization of silanol groups (pK_a ~ 4.9) and thus it features pH dependency. Also, it is prone to instability due to adsorption of cationic and hydrophobic compounds. This issue can be solved either by harsh rinsing procedures before an analysis or coating of the silica surface with appropriate chemistry. Physically adsorbed coatings are easily applicable for such task. In this report, we present two new physically adsorbed coatings which belong to group of polymeric ionic liquids. This group of polyelectrolytes has certain advantages, including a wide range of possible designs and change of solubility depending on a counterion selection. A few works utilizing PILs in CE either as dynamic, physically adsorbed or

permanent coatings were recently reviewed in [1]. Two in lab synthesized PECs are evaluated as physically adsorbed coatings for CE in this work featuring two separation applications.

2 Experimental

Solutions containing 1 mg·ml^{-1} of PMOTAI (M_w ~ 51 900) and PIL-1 (M_w ~ 25 100) [2], respectively, were used for coating the capillary. Each capillary was pre-conditioned by 10 min rinse with 0.1 M NaOH, followed by a 10 min water rinse. The coating solution was then washed through the capillary for 10 min, after which the flow was stopped and the PEC solution was left inside the capillary for 10 min. Finally, the coating solution was rinsed out with BGE for 10 min. A recoating process was also optimized and it was composed of the same steps but time was shortened to 1 min. Agilent 7100 CE system from Agilent Technologies (Santa Clara, CA, USA) equipped with a UV-Vis DAD was used for all CE analyses. The adsorbed PEC layers was thickness and coating dynamics were analyzed by an impedance-based quartz crystal microbalance (QCM-Z500, KSV, Biolin Scientific Oy, Finland) utilizing silica coated quartz crystal sensor.

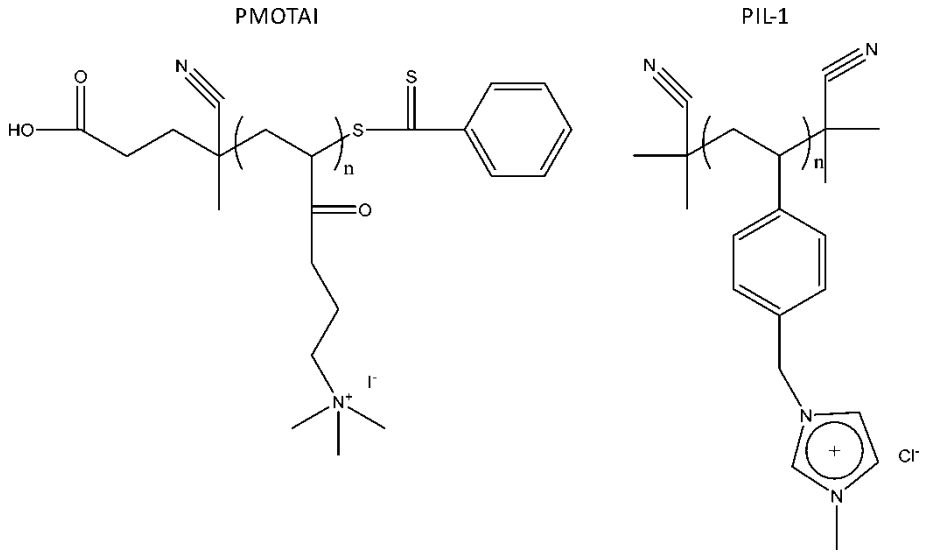

Fig. 1. Structures of polyelectrolytes.

3 Results and Discussion

A series of CE analyses were used to determine the stability of the adsorbed PECs layers. In a freshly coated capillary with PMOTAI and PIL-1 the EOF was -4.6·10^{-8} to -4.1·10^{-8} m^2·V^{-1}·s^{-1}, respectively. Based on the stability study, five CE runs were always done before a short one minute flush with the coating solution was used for a coating regeneration. The pH stability was tested with sodium phosphate buffer in pH range 3 – 11. The EOF decreased with increase of pH from 4 to 8, after which the EOF leveled out. We observed instability of the EOF with both coatings at high basic pH value demonstrated by a faster decrease of the EOF. The thickness of the both coatings measured by QCM-D was below 1 nm and the obtained value was further

used for phase ratio calculation. CE was then used in the open tubular capillary electrochromatography mode to determine retention factors of six alkyl benzoates. Calculated distribution constants were close to octanol/water system values. This suggests a hydrophobic nature of both PECs coatings. In addition, higher distribution constants were observed with PIL-1, which might be due to π-π interaction between the aromatic cores in PIL-1 and the alkyl benzoates. The separation of a mixture of five β-blockers was obtained at pH 3. Hydrolytic products of [DBNH][OAc] were analyzed in acidic conditions to stop further hydrolysis. Both tested coatings gave baseline separation of all three compounds in less than 5 minutes at pH 3.

4 Conclusions

The new polyelectrolytes proved to be suitable for semi-permanent cationic coating in CE. They feature an easy capillary coating procedure and good EOF stability at pH values below 10. A short recoating step was used to maintain a stable EOF. Moreover, the hydrophobicity of the coating enabled separation of compounds with log $P_{o/w}$ values higher than two.

Acknowledgement

Financial support from Magnus Ehrnrooth Foundation (SKW) and the Academy of Finland projects 266342 (SKW), 276075 (SKW), and 137053 (TV) are gratefully acknowledged.

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COMPARISON OF IONISATION PROPERTIES OF AETMA-LABELED SACCHARIDES WITH COMMON LABELS

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Summary

We have tested (2-aminoethyl)trimethylammonium (AETMA) as a label for analysis of oligosaccharides by capillary electrophoresis with electrospray (ESI) mass spectrometry detection and compared its performance to taurine, 2-aminobenzoic acid, 2-aminobenzamide and 8-aminopyrene-1,3,6-trisulfonic acid. The AETMA-labeled saccharides were provided higher ionization signals in positive mode than

native sodium or ammonium adducts and equal or higher signals than saccharides labeled by the more commonly used labels tested in this study.

1 Introduction

The analysis of oligosaccharides and especially the analysis of glycans play a significant role in current bioanalytical chemistry. Due to the complex structure of the glycan samples the analysis usually requires an efficient separation technique. Capillary electrophoresis with mass spectrometry detection represents an emerging powerful tool for this task. Labeling of oligosaccharides by quaternary amine (AETMA) with permanent positive charge allows highly efficient separation in capillary electrophoresis as well as ESI detection by mass spectrometry in positive ionization mode with higher sensitivity [1].

2 Experimental

The saccharides were labeled by AETMA via reductive amination under acidic conditions in a mixed water/methanol solution using 2-picoline borane as the reductive agent as described in our previous work [2]. The labeling by taurine was performed in alkaline solution and labeling by the more commonly used labels (2-aminobenzoic acid, 2-aminobenzamide and 8-aminopyrene-1,3,6-trisulfonic acid trisodium salt) was performed under non-aqueous acidic conditions with sodium cyanoborohydride as the reductive agent. Isomaltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose and hydrolyzed dextran ladder were selected as the model sample. Labeled samples were diluted by 50% methanol with 1% formic acid for positive mode or by 50% methanol with 1% ammonium for negative mode. The ESI/MS analysis was performed with direct infusion using an ion trap mass spectrometer (Amazon SL, Bruker, Bremen, Germany). Mass spectrometer conditions were optimized individually for each type of label. The ionization intensities of labeled standards were measured individually and compared with one another. Experiments with CE-MS were performed on Agilent CE ESI-MS Sprayer Kit (G1607A, Agilent Technologies) with PAA-coated fused silica separation capillary. Background electrolytes for capillary electrophoresis were 100 mM acetic acid (pH = 2.9) for positive mode and 10 mM ammonium acetate (pH = 8.5) for negative mode. Sheath liquid composition was the same as in the infusion experiments.

3 Results and Discussion

We have compared ionization properties of saccharide standards labeled by AETMA, taurine, 2-AA, 2-AB and APTS in positive and negative ionization modes of the mass spectrometer. The taurine was used as a negative analogue to AETMA for its similar structure differing only by substitution of the quaternary amine for a sulfonic group. Unlabeled oligosaccharides were used as standards for comparison of the ionization intensity, and detected as sodium or ammonium adducts. In comparison to the native (sodium adducted) oligosaccharides, the AETMA-labeled species had approximately ten times higher intensity in the positive ESI mode. The taurine-labeled saccharides

measured in negative mode provided approximately ten times lower signal intensity compared to sodium adducts of native saccharides in positive mode. Neither AETMA nor taurine are commonly used as labels for saccharides. Thus we have compared the measured ESI/MS intensities to the signals obtained with other labels, namely, 2-AA, 2-AB and APTS-labeled measured in positive or negative ESI mode. 2-AA- and 2-AB-labeled saccharides provided similar ionization signals as AETMA-labeled saccharides and ten times higher ionization intensity than sodium adducts of native saccharides. APTS-labeled saccharides provided ten times higher intensity than taurine-labeled saccharides and approximately ten times lower intensity than sodium adducts of native saccharides. These measurements confirm that AETMA is suitable as a label for sensitive analysis of saccharides providing high ionization signal in positive ESI mode. AETMA-labeled neutral saccharides were successfully separated by capillary electrophoresis migrating as one singly charged cations and were detected by ESI/MS as doubly or triply charged ions, depending on the oligosaccharide chain length. This fact made interpretation of MS records more comfortable because there was not required of wide range of m/z.

4 Conclusions

In this work, we have presented AETMA as a rarely used label for saccharides which seems to be promising for sensitive analysis by CE-MS. The AETMA-labeling enabled capillary electrophoretic separation and sensitive mass spectrometric detection of saccharides. This approach allowed measurement of labeled saccharides with comparable sensitivity to 2-AA and 2-AB and approximately ten times higher sensitivity in comparison to sodium adducts of native saccharides and measurement of common labels in negative mode of mass spectrometer.

Acknowledgement

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ONLINE CONNECTION OF FREE-FLOW ISOTACHOPHORESIS CHIP TO AN ELECTROSPRAY IONIZATION MASS-SPECTROMETER

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Summary
We present the online coupling of a free-flow isotachophoresis (FFITP) chip to an electrospray ionization mass spectrometer (ESI-MS) for continuous online analysis without extensive sample preparation. In comparison with batch electrophoretic techniques, FFITP allows for the continuous injection of target analytes into the MS, making detection independent of the separation time domain.

1 Introduction

Free-flow-electrophoresis techniques are used for continuous electrophoretic separations by applying an electric field perpendicular to the buffer- and sample flows [1, 2], with FFITP capable of concurrently focusing a target analyte while removing interferences. The online coupling of FFITP to an ESI-MS will provide access to structural information of the target analytes by decoupling the detection from the separation timeframe because the electrophoretic separation takes place perpendicular to the flow direction [3]. This decoupling can be beneficial for monitoring (bio)chemical changes and/or for extensive MS^n studies in proteomics.

2 Theory

Glycolic and citric acid were selected as model analytes in combination with Alexa fluor 488 (Alexa) as fluorescent tracer, whilst fluorescein was used as model contaminant. Based on effective mobilities, formic acid was chosen as the leading electrolyte (LE), and propionic acid was as terminating electrolyte (TE) so that, when an electric field is applied Alexa, citric and glycolic acid will stack between LE and TE whilst the lower mobility fluorescein would dissipate into the TE, as modeled in SIMUL (Figure 1) [4].

3 Experimental

3.1 Chemicals

10 mM Formic acid was adjusted to pH 4.29 with ammonium hydroxide and used as LE, 7mM propionic acid (pH 3.55) was used as TE. Samples contained 1 mM fluorescein, 1 mM citric acid and 1 mM Alexa, and 1 mM glycolic acid was added for the MS scanning study. All chemicals were purchased from Sigma Aldrich (Germany) with the exception of Alexa fluor 488, which was purchased from Life Technology (Germany).

3.2 Experimental setup

The FFITP device was mounted on an Axiovert inverted microscope. LEDs were used for sample illumination. Imaging was performed using the 5X objective lens and color CCD camera with the LED intensity adjusted to 1 A. The fluorescence intensity measurements were performed using a single point detector made using a 50X objective lens and photomultiplier tube (PMT) with the gain set by 0.5 V bias. Samples and buffer were injected through a neMESYS Low Pressure Syringe Pump system comprised of five syringe modules. To connect the tubing from the syringes to the device, Bondend port connectors were bonded to the glass chip using epoxy. A fused silica capillary with an outer diameter of 360 µm and inner diameter of 100 µm was used to connect the FFITP chip to the ESI interface of the 1100 LC/MSD mass spectrometer. The potential difference was applied using a HVS448 High Voltage Sequencer (Labsmith, USA).

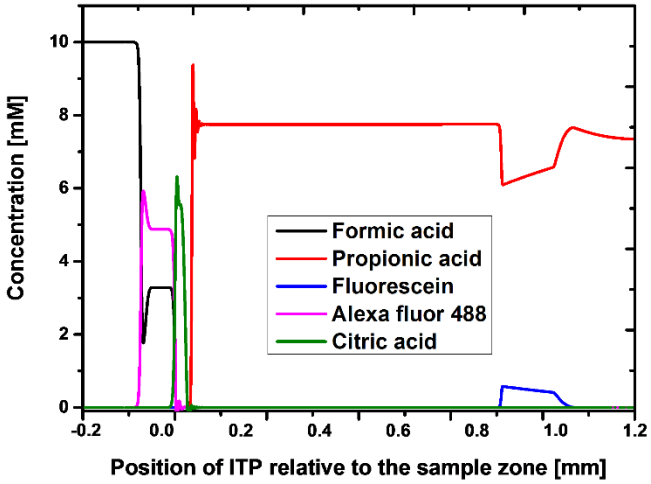

Fig.1. 10 mM of formic acid adjusted to pH 4.29 with ammonium hydroxide was used as leading LE, and 7 mM propionic acid at pH 3.55 was used as TE. The sample consist of 50 µM fluorescein, 100 µM citric acid and 100 µM Alexa fluor 488. By applying an electric field fluorescein dissipates into the TE, whilst citric acid and Alexa fluor 488 will be concentrated at the leading buffer side.

4 Results and Discussion

The microscope images in Figure 2(a) show the bright focused Alexa zone separated from the faint dissipating fluorescein when the voltage is applied. Hydrodynamic control was used to move the ITP zones past the detection point, with Figure 2(b) showing the photomultiplier tube (PMT) trace with and without applied fields, showing the concentration of Alexa by factor of 40 with removal of the fluorescein.

Figure 3(a) shows the relative abundance of the MS signal for citric acid and fluorescein with and without applied electric field, with an increase in citric acid combined with a drop in fluorescein. More significant enhancements are expected

when lowering analyte concentration moving to peak mode ITP. Hydrodynamic flow control was used to direct separated analyte zones into the MS, enabling scanning of the separation space by MS. Figure 3(a) shows the MS data output as function of the TE flow rate, with the higher mobility citric acid being detected before the slower glycolic acid (Figure 3b).

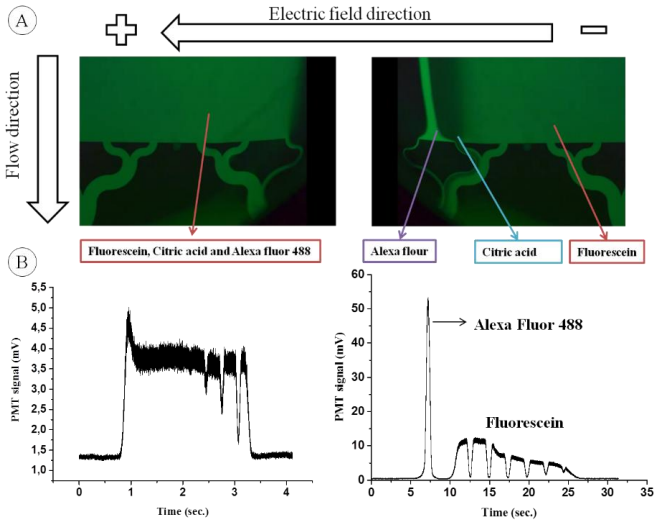

Fig. 2. (A) Microscope image of the FFITP device where the left panel shows the chamber in absence of an electric field and the right panel shows the concentrated line of Alexa separated from the fluorescein in the electric field.(B) PMT signal output where the left panel is without and the right panel is with electric field applied.

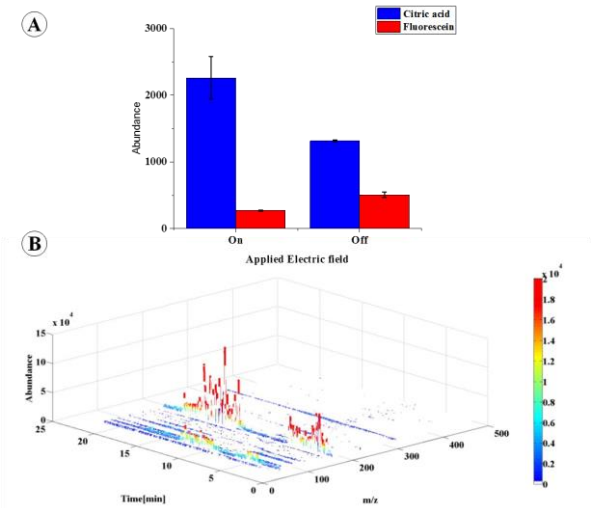

Fig. 3. (A) Continuous data collection was done for more than 50 minutes, while turning on and of the electric field. By applying an electric field the abundance of citric acid increased from 1250 to 2300, and the abundance of fluorescein decreased from 500 to 280. (B) Changing the inlet flow rates enabled guidance of specific flow lines into the MS.

5 Conclusions

We have demonstrated the coupling of FFITP with ESI-MS for simultaneous concentration of target analytes and sample clean-up. Conducting ITP in the free flow format allows for continuous injection into the MS for monitoring purposes or extended MS studies in proteomics [5].

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Abstracts of poster presentations

P1 CHARACTERIZATION OF LIGNIN SAMPLES ISOLATED FROM BEECH USING KLASON METHOD BY SIZE-EXCLUSION CHROMATOGRAPHY WITH NARROW-BORE COLUMNS

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Summary
A size-exclusion chromatography method using narrow-bore (3.5 mm i.d.) columns filled with methacrylate based gels (Spheron 40 and Spheron 100), fluorescence detection (FLD), and mixture of N,N-Dimethylformamide and phosphate buffer (pH 3.00) as mobile phase was used for the characterization of Klason lignin samples isolated from beech.

1 Introduction

Wood is composed of many chemical components, primary extractives, carbohydrates, and lignin. Lignin is the second most abundant biological macromolecule, and comprises 15 – 25% of the dry weight of woody plants. Although lignin is necessary to trees, it is undesirable in papermaking process and is removed by kraft pulping and bleaching processes, resulting in waste known as black liquor [1]. For the characterization of black liquor composition of macromolecules and small molecules after delignification processes, size-exclusion chromatography (SEC) is often used.

Size-exclusion chromatography or high-performance size-exclusion chromatography (HPSEC) is a liquid chromatographic technique for measuring molar mass distributions (MMDs) of natural and synthetic polymers and macromolecules, and is normally used as an analytical procedure for separating molecules by their difference in size and to obtain molar mass averages. The information generated by SEC reflects the molecular size distribution (MSD), which is converted into a MMD using calibration curve [2, 3].

Currently, there is a strong trend in liquid chromatography to reduce the size of the chromatographic column. Traditionally, in SEC, column internal diameters of 7 - 8 mm i.d. are employed, although in recent years, narrow-bore (3 - 6 mm i.d.) columns have become more often used. Most narrow-bore columns have inner diameter of 4.6 mm i.d., directly between the standard analytical columns and the microbore columns (< 3 mm i.d.) [4].

As opposed to standard SEC columns, narrow-bore columns offer sharper peaks, which are a direct result of the lesser radial dispersion, due to the narrower column width. The increased peak sharpness may compensate for the loss of resolution resulting from the non-optimal flow rates. To maintain the same linear velocity through the column, the volume flow rate must be reduced by a factor of about two or three, which results in significantly lower solvent consumption. The use of narrow-bore columns may make it also easier to adequately control the temperature in the analysis of polymers that cannot be dissolved at room temperature [2, 5, 6].

A disadvantage of narrow-bore columns is that the band-broadening effects are more severe because the contribution of the outside column volume in connecting tubing and detectors is related to smaller column volume and thus it is relatively more significant. To use the potential of narrow-bore columns it is necessary to minimize the system dispersion. Another drawback of narrow-bore column is its reduced capacity due to smaller amount of stationary phase particles and thus smaller surface area of the column [6].

Miniaturized SEC columns are desirable if SEC is to be used as first-dimension separation in comprehensive two-dimensional liquid chromatography (SEC × LC), when preparing various molar mass fractions for further use. However, it is not necessary to keep this order, when reversed-phase high-performance liquid chromatography (RP-HPLC) is used for the fractionation of the sample and SEC, as the second dimension, is used for the characterization of each RP-HPLC fraction [2, 7].

The aim of this work was to characterize lignin samples isolated from beech using Klason method on narrow-bore columns filled with hydroxyethylmethacrylate gels Spheron 40 and Spheron 100.

2 Experimental

2.1 Material

Lignin samples were isolated by Klason method [8] from beech wood. This method is based on hydrolysis and dissolution of cellulose and hemicellulose isolated from wood or wood pulp by sulfuric acid with decreasing concentration from 72% to 3%. Isolated Klason lignin was subsequently washed and dried [9]. Prior the analysis, lignin samples were dissolved in N,N-Dimethylformamide (DMF) for several days, however dissolution of samples in DMF was limited, thus 6% LiCl was added to the solvent. Lignin samples were prepared at concentration of 500 mg L^{-1}.

2.2 Instrumentation and chromatographic conditions

A Merck-Hitachi LaChrom chromatographic system equipped with L-7100 gradient pump, L-2000 autosampler, L-7480 fluorescence detector, L-7300 column termostat, and D-7000 HSM software was used. Klason lignin samples were characterized on two narrow-bore columns (250 x 3.5 mm i.d.) filled with hydroxyethylmethacrylate (HEMA) gel Spheron 40 with relative molar mass exclusion limit range from 20 000 to 60 000 and particle size < 25 μm, and Spheron 100 with relative molar mass exclusion limit range from 70 000 to 250 000 and particle size 25 – 40 μm, according

to manufacturer [10]. Mobile phase consisted of 99% (v/v) DMF and isocratic elution at ambient temperature was used.

3 Results and Discussion

In available publications authors usually use as a solvent and mobile phase dimethyl sulfoxide (DMSO) or N,N-Dimethylacetamide (DMAc) [11, 12]. In our work N,N-Dimethylformamide (DMF) was chosen as mobile phase, because of its excellent solvation properties for polymeric substances and macromolecules, ability to form hydrogen bonds and low volatility [13, 14, 15].

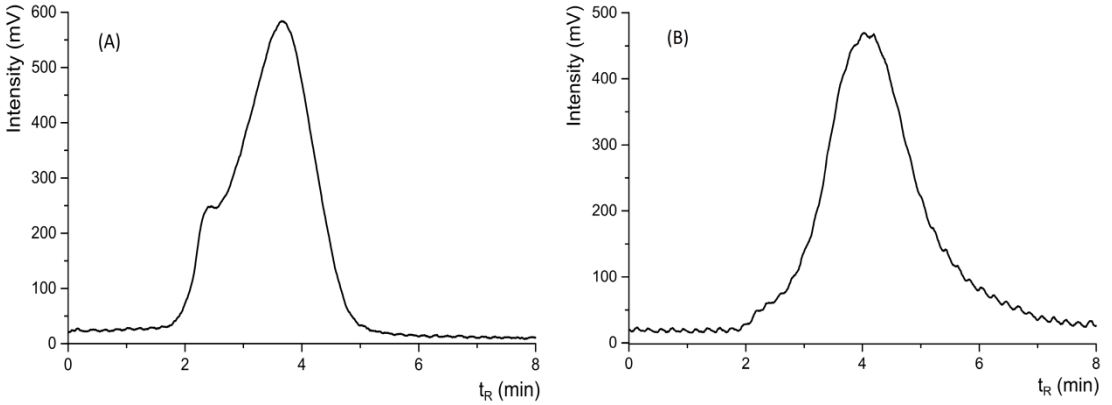

Fig. 1. SEC profiles of beech lignin sample characterized on gel Spheron HEMA 40 and dissolved in (A) DMF and (B) 6%LiCl/DMF (injection volume (A) 3 µl, (B) 15 µl).

As shown in Fig. 1., when using 6% LiCl/DMF as a solvent, the share of non-excluded molecules is higher, what is probably associated with the suppression of association phenomena of lignin samples by addition of lithium salt.

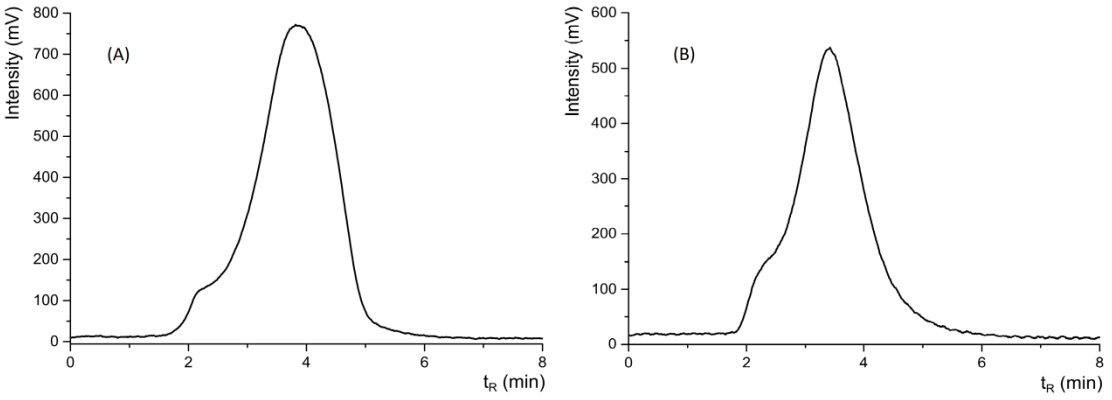

Fig. 2. SEC profiles of beech lignin sample characterized on gel Spheron HEMA 100 and dissolved in (A) DMF and (B) 6%LiCl/DMF (injection volume (A) 3 µl, (B) 15 µl).

When using gel Spheron HEMA 100, the ratio between excluded and non-excluded molecules is similar (Fig. 2.).

As shown in Fig. 1. and Fig. 2., the intensity of fluorescence signal is lower when using mixture LiCl/DMF as a solvent, what can be related to quenching of fluorescence signal of samples in presence of LiCl.

4 Conclusions

A fast size-exclusion chromatography method using narrow-bore columns filled with hydroxyethylmethacrylate gels and N,N-Dimethylformamide was used for the characterization of lignin samples isolated from beech wood by Klason method.

Acknowledgement

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P2 AN AUTOMATED CAPILLARY ELECTROPHORESIS IN A SEPARATION SYSTEM WITH ENHANCED SAMPLE LOADABILITY

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Summary
A unique sample injection module for automated electrophoretic equipment operating in a single column mode with enhanced separation capacity was designed. The injection device was made of polymethylmethacrylate with a fixed sample volume (1 μL) and a sample plug length of 5 mm. A reasonable separation efficiency for the anions of organic acids (benzoate, sorbate and ascorbate) was achieved under the eliminated hydrodynamic and electroosmotic flow during the CE separation and field-amplified sample stacking applied. Analytical parameters for the model analytes using this type of sample introduction were comparable to those on similar devices operating in a manual regime. The contents of benzoate and sorbate determined in two real samples – soft drinks by external calibration and standard addition methods were in a good agreement.

1 Introduction

At present, CE separations are performed, almost exclusively, in the capillaries with inner diameter (ID) less than 100 μm. Although the capillaries of such dimensions provide a very high separation efficiency, the separation capacity is limited (sample volume up to 10 nL) [1], which resulting to the need for very sensitive detectors. This could be partially solved by the use of appropriate sample pre-concentration techniques combined in-line or on-line with the separation, e.g. extraction techniques or techniques based on the electrophoretic principles (transient ITP, field-amplified stacking and dynamic pH junction) [2-4].

In the past, it was shown that CE separations performed in the capillaries with the IDs of 200-300 μm are not associated with a significant reduction of separation efficiency by heat generation [1, 5-10] when the separation conditions are chosen carefully. An enhanced sample loadability (100-500 nL volume) and a longer path length of light beam in a case of photometric detection result in a very good concentration LOD [1, 6, 7, 10]. On the other hand, a movement of the electrolyte solution in the CE systems equipped with capillary of larger ID has to be prevented by a barrier, because the electrolyte solution is not hydrodynamically stabilized by wall effect. A fully automated version of the CE device with large-bore capillaries, developed at our department, is used mainly for on-line combination of ITP and CE [11-13].

This work was aimed at the development and testing of the new sample injection module for CE carried out in the automated hydrodynamically closed electrophoretic equipment and provided with a capillary of 300 μm ID.

2 Experimental

An automated electrophoretic analyzer EA202A (Villa-Labeco, Slovakia) was used in the single column mode (Fig. 1).

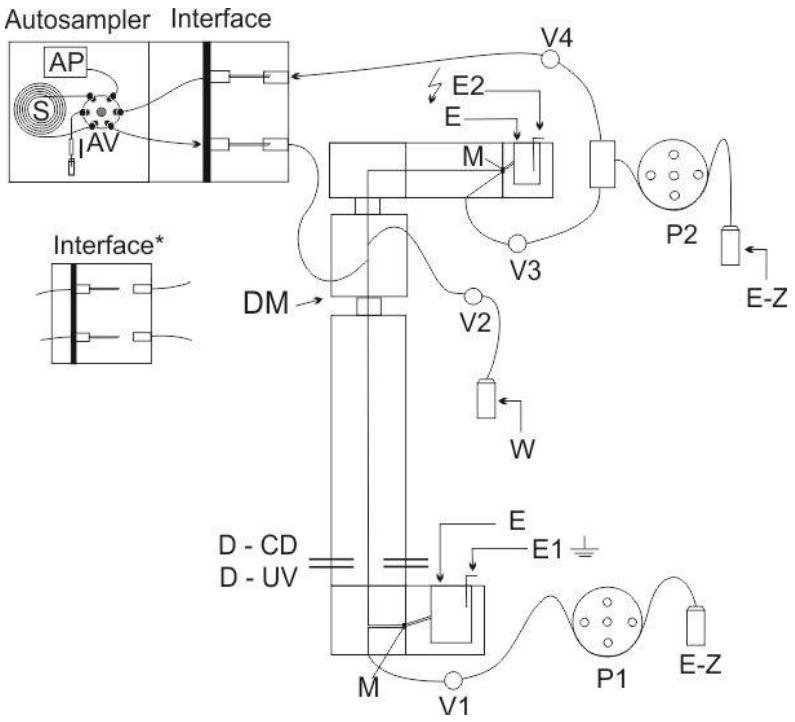

Fig. 1. The scheme of automated electrophoretic analyzer. Autosampler: S – sample loop, AP – pump, I – syringe, AV – 6-way valve. Separation and electrolyte unit: V1-V4 – pinch valves, E1-E2 – electrodes, DM – injection module, W – waste, D-UV, D-CD – absorbance and conductivity detection cell, E – electrode vessel, M – membrane, column – fused silica capillary (300 μm ID), EZ – electrolyte vessels, P1-P2 – peristaltic pumps, Interface* – position under separation.

3 Results and Discussion

The channels in the injection module were designed in order to allow the field-amplified sample stacking and to minimize fluctuations of sample volume loaded. The preliminary experiments, performed with colored sample (amaranth solution), showed that the sample plug exceeds space between the inlet and outlet channels in both directions. Reproducible sample injection, assessed also by visual inspection (Fig.2), was reached by modifications made on the fluidic part of the automated CE device.

Fig. 2. The sample injection module made of polymethylmethacrylate.

The evaluation of the new sample injection module employed in this work was performed on the CE separations of the model mixture of benzoate, ascorbate, sorbate and sulfate using the carrier electrolyte at pH = 5.2 [14]. As noted above, the elimination of hydrodynamic and electroosmotic flow during the separation, as well as the injection of the sample solution of low conductivity was essential in term of separation efficiency. We observed that a residual pressure due to a leakage in the closure of the separation system (pinch solenoid valve) reduced the number of theoretical plates by a factor of 10. A similar decrease of the separation efficiency was observed in an absence of field amplified sample stacking (model sample prepared in the carrier electrolyte).

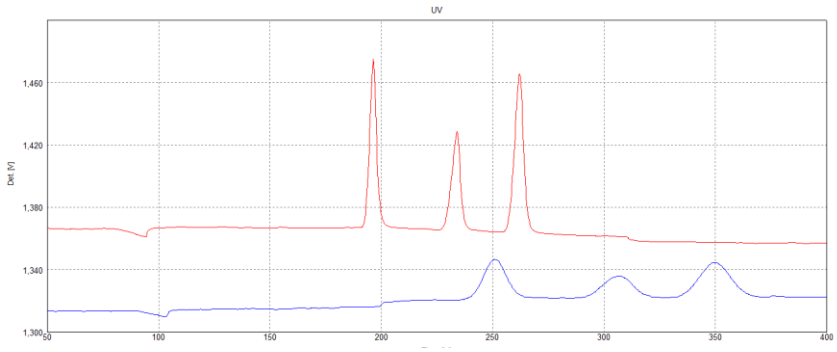

Fig. 3. CE separation of benzoate (30 µM), ascorbate (120 µM) and sorbate (30 µM) without (upper) and with hydrodynamic counter-flow (lower). UV absorbance detection was performed at λ = 220 nm.

The parameters of the calibration lines measured four times within one month were reproducible. The concentrations of benzoate and sorbate were determined in two different soft drinks – Vinea and Marka (Table 1). The concentrations of the analytes calculated by external calibration and standard addition methods were in a good agreement.

Table 1. Determination of benzoate and sorbate in soft drinks.

Sample	Analyte	Mean ± standard deviation (µmol/L), *n = 3*	
		External calibration	Standard addition
S1	Benzoate	1022.7 ± 2.6	1125.1 ± 7.1
	Sorbate	1043.5 ± 46.9	1061.3 ± 30.3
S2	Benzoate	587.6 ± 32.9	563.9 ± 20.3
	Sorbate	1297.6 ± 25.7	1317.8 ± 67.7

4 Conclusions

The employed sample injection module extends the application possibilities of the automated electrophoretic analyzer with the large bore capillaries for single column CE separations.

Acknowledgement

The research was supported by the Slovak Research and Development Agency (APVV-0259-12) and the Scientific Grant Agency (VEGA 1/0342/15).

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P3 EFFECTS OF COMPLEXATION OF BUFFER CONSTITUENTS WITH CHARGED CYCLODEXTRINS

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Summary
Cyclodextrins (CDs) are frequently used in capillary electromigration methods as additives to background electrolyte (BGE). Presence of CDs in BGE offers additional interactions to analytes thus selectivity of separation systems can be enhanced, neutral analytes can be mobilized or chiral separation can be achieved. However, these additives can complex also with buffer constituents[1]. This type of interaction can significantly change some of the background electrolyte properties[2]. Moreover, when charged CDs are used, the ionic strength of background electrolyte is increased due to the high number of charges on CD molecule. This work is focused on revealing complexation between charged CDs and proposing safe *i.e.* noninteracting buffers for electrophoretic measurements with charged CDs.

Equations for calculations of pH changes in case of complexation of charged CDs with weak electrolyte buffer component were derived. Changes of buffer pH after addition of charged CD were measured for 5 CDs and 8 selected buffering electrolytes (acetic acid, formic acid, benzoic acid, MES, CHES, MOPS, TAPS, tricine). Additional electrophoretic measurements were carried out to confirm the results obtained by means of pH measurements. Further, the concept of IS marker was utilized to qualitatively explain effect of ionic strength changed by addition of charged CD.

Among 8 tested buffer constituents the TAPS, MOPS and MES were proved as a noncomplexing "safe" buffer constituents for all charged CDs tested. Benzoic acid and CHES showed to be strongly complexing will all charged CDs tested except for sulfated α-CD due to size of its cavity. Acetic and formic acid slightly interacts with randomly sulfated β- and γ-CDs tested. Tricine showed to be interacting with all charged CDs tested.

Acknowledgement
The authors gratefully acknowledge financial support of the Grant Agency of the Czech Republic, Grant No. P206/14-19278P and Grant No. 15-18424Y.

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P4 COMPARISON OF ANTIOXIDANT AND COLOUR CHARACTERISTICS OF DIFFERENT TYPES OF MEDICAL PLANTS ASSESSED BY MODERN SPECTROSCOPIC TECHNIQUES

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Summary
This study is focused on comparison of antioxidant and colour properties of different types of herbs originating from two botanical institutions by using modern spectroscopic techniques.

1 Introduction
Research on antioxidant properties of medicinal plants has gained enormous popularity in recent years. Medicinal plants are being viewed as easily available source of antioxidants. Antioxidant effect of herbs is mainly attributed to phenolic compounds (flavonoids and phenolic acids) and carotenoids, but the role of non-flavonoid antioxidants is widely discussed, as well. Besides antioxidant activity flavonoids and carotenoids contribute to the creation of colour in herbs [1−3]. The aim of this study was to compare the antioxidant and colour characteristics of various types of herbs originating from two different locations in the Czech Republic.

2 Experimental
2.1 Examined samples
Six varieties of medical plants: lavender (*Lavandula augustifolia* LA), pot marigold (*Calendula officinalis* L. CO), St John's wort (*Hypericum perforatum* HP) clary sage (*Salvia sclarea* SS), lemon balm (*Melissa officinalis* MO) and galega (*Galega officinalis* GO) were analysed. Herbs were harvested in their full ripeness in the Medicinal Herbs Centre (MHC) Brno, Czech Republic and the Faculty of

Horticulture, Mendel University (FHM) Lednice, Czech Republic, during June 2015. After collecting, the samples were frozen at −18°C and stored until analysis.

2.2 Preparation of extracts

Exactly 0.5 g of respective homogenized herb sample was placed into centrifuge tubes, 20 ml of 50% ethanol was added and the mixture was shaken on a shaker for 60 min at 150 rpm at 20°C, then centrifuged at 10 000 rpm at 20°C for 10 minutes, filtered through a filter into a dark vial. Extracts were stored in the refrigerator at 5°C during the analysis. Extracts were prepared in duplicates, every sample was analysed two times (number of repetitions, n = 4).

2.3 UV-VIS experiments

The entire UV-VIS experiments were performed using UV-VIS-NIR spectrophotometer Shimadzu 3600 with accessories. Total phenolic content (TPC) was determined applying the Folin-Ciocalteau modified method [4]. Total flavonoids content (TFC) was determined by the modified method using 2-aminoethyl-diphenylborate reagent [4]. Colour coordinates L*, a* and b* of extracts were evaluated directly from the measured spectra by means of ColourLite Panorama Shimadzu software using the D65 day light illuminant as the light source and 10° standard observer.

2.4 EPR experiments

All the experiments were performed using a portable X-band EPR spectrometer e-scan with accessories. $ABTS^{\bullet+}$ radical-scavenging activity of samples was tested by the method previously described by Tobolková et al. [4]. Radical-scavenging activity was expressed as a TEAC (Trolox equivalent antioxidant capacity) value. The direct antioxidant activity of samples, i.e., their capability to scavenge the $^{\bullet}OH$ radicals generated via thermal decomposition of $K_2S_2O_8$ radical initiator in the presence of DMPO spin trap was assessed in accord with procedure previously published by Tobolková et al. [4]. Antioxidant activity of samples was expressed as % RS value (radical scavenging activity, % of extinguished radicals).

3 Results and Discussion

From the results of TPC assay presented at Figure 1, some differences in polyphenol content between the samples originating from MHC and FHM are obvious. Generally, samples from FHM were characterized by the higher levels of TPC, TFC and $TEAC_{ABTS^{\bullet+}}$ than those originating from MHC, however this difference is in all the cases considered to be statistically insignificant ($P>0.05$). TPC values of individual herbs, decreased for samples of both institutions in the following order: HP > GO > MO > SS > LA > CO. Statistical analysis confirmed similarities also for all other selected parameters of herbs originating from both locations. Locations are 55 km far from each other, so similar climatic and soil conditions can be expected. Minor differences in selected parameters may result from slightly different weather conditions and manipulation with herbs during harvest and post-harvest treatment [5].

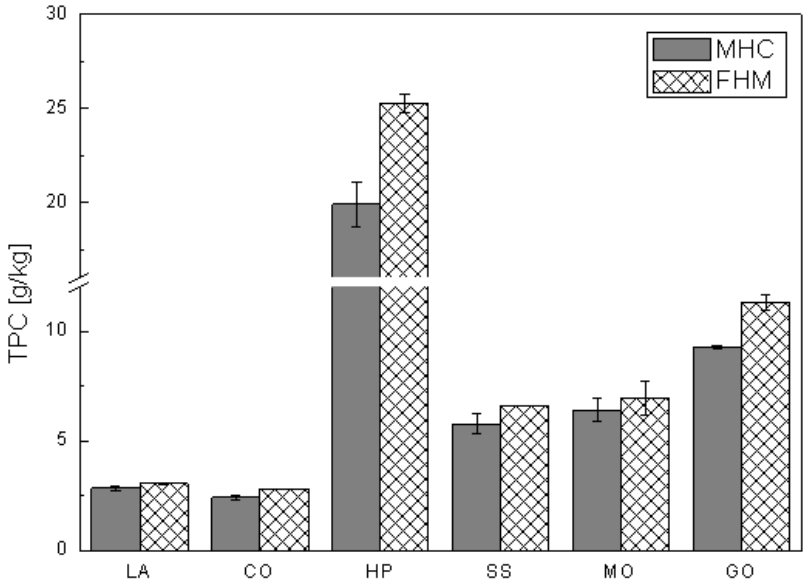

Fig. 1. The average values of TPC (g/kg) determined in ethanol extracts of selected herbs. Samples specification is given in the experimental part.

Correlation analysis also confirmed that the content of polyphenols and flavonoids correlate positively with total antioxidant activity expressed as $TEAC_{ABTS^{\bullet+}}$ ($P_{TPC/TEAC}$=0.977, $P_{TFC/TEAC}$=0.693). These results confirmed the fact that polyphenols and flavonoids are mainly responsible for antioxidant properties of herbs [1]. Hydroxyl radicals and carbon-centred radicals were identified as being dominantly generated in ethanol extracts of selected herbs in the presence DMPO/$K_2S_2O_8$. In case of colour characteristics, L* values of all extracts reached maximum - 100, indicating that the herbal extracts are pure with a distinctive bright tone. For majority of the studied herbs (except St John's wort, which contains red pigment hypericin), negative values of colour coordinates a* were obtained, indicating dominant representation of green colour in the extracts, probably as the result of the presence of chlorophyll pigments. On the other hand, positive values of b* proved the dominant yellow colour in extracts, affected by the presence of flavonoids and carotenoids [3].

4 Conclusions

Non-significant differences in the monitored characteristics of herbal extracts originating from two different locations were determined, probably caused by similar climatic and weather conditions in both places. Additional experiments are in progress, focused on detailed analysis of radical type/concentration formed in the samples. The solvent-dependent effects are also further studied.

Acknowledgement
This contribution is the result of the project "Establishment of a HiTech Centre for Research on Formation, Elimination and Assessment of Contaminants in Food" supported by the Research & Development Operational Programme funded by the ERDF. The Medicinal Herbs Centre in Brno, Czech Republic and the Faculty of Horticulture, Mendel University in Lednice, Czech Republic, are gratefully acknowledged for samples provision and kind cooperation.

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P5 SYSTEM FOR FAST ANALYSIS OF EXPLOSIVES IN THE ENVIRONMENT

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Summary
Detection of explosives is the subject of many investigations by law enforcement agents and forensic scientist. The identification and discrimination of evidence taken from crime scenes is a common practice in forensic investigations. The higher demands on simple and fast determination of hazard materials or strange objects are required in connection with worldwide often repeated terrorist activities.

1 Introduction
Exlosives belong to the very specific group of environment contaminants. Their presence is typically located in the areas after military training and wartime activities. The other sources of contamination include the manufacturing, testing and disposal of explosives [1,2].

2 Experimental

Present paper describes the fast and sensitive method for the analysis of explosives in the environment using the novel portable device [3], that is assembled from the automated microcolumn liquid chromatograph [4,5], photolytic converter and miniaturized chemiluminescence detector with selective response [6].

The device is able to determine selectively nitramine- and nitroester- and most of nitroaromates-based explosives as well as inorganic nitrates at trace concentrations in water or soil extracts in less than 8 minutes without previous preconcentration procedures. Nitrates as well as separated explosives are on-line photolytically converted to peroxynitrite and then detected by the chemiluminescence reaction with the alkaline solution of luminol.

3 Results and Discussion

The efficiency of explosive extraction was measured with spiked samples of soil – NG, EGDN and Tetryl (1×10^{-6} M), TNT, 2,4-DNT, 2,6-DNT, TNB and DNB (1×10^{-4} M), NB (1×10^{-3} M), DMDNB and sodium nitrate (5×10^{-6} M) – and was determined to be in range 89–96 % for all analytes. The repeatability of the method was determined by repeated injections of standard samples during 4 hours. RSD of retention time was 2.9–5.6 %. The method allows to analyse all chosen compounds in less than 8 minutes. We analysed 14 compounds (HMX, RDX, PETN, NG, EGDN, TNT, 2,4-DNT, 2,6-DNT, TNB, DNB, NB, Tetryl, DMDNB and nitrate) according list in EPA8330 method. Limits of detection of compounds of interest are in the range of concentrations from 5.0×10^{-9} M to 8.0×10^{-5} M for a signal-to-noise ratio of 3. Limits of quantification are in the range of concentrations from 1.7×10^{-8} M to 2.7×10^{-4} M for a signal-to-noise ratio of 10.

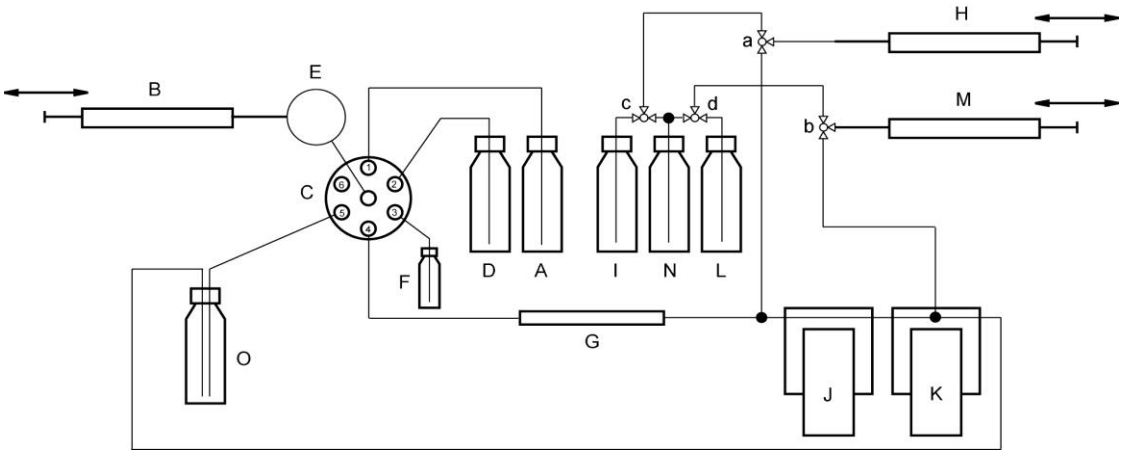

Fig. 1. Scheme of the device: A – strong mobile phase reservoir, B – syringe with mobile phase, C – selector valve, D – weak mobile phase reservoir, E – sample loop, F – sample, G – capillary column, H – syringe with SMA solution, I – SMA reservoir, J – UV converter, K – detector, L – luminol reservoir, M – syringe with luminol solution, N – deionized water reservoir, O – waste; a, b, c and d – 3-ways valves.

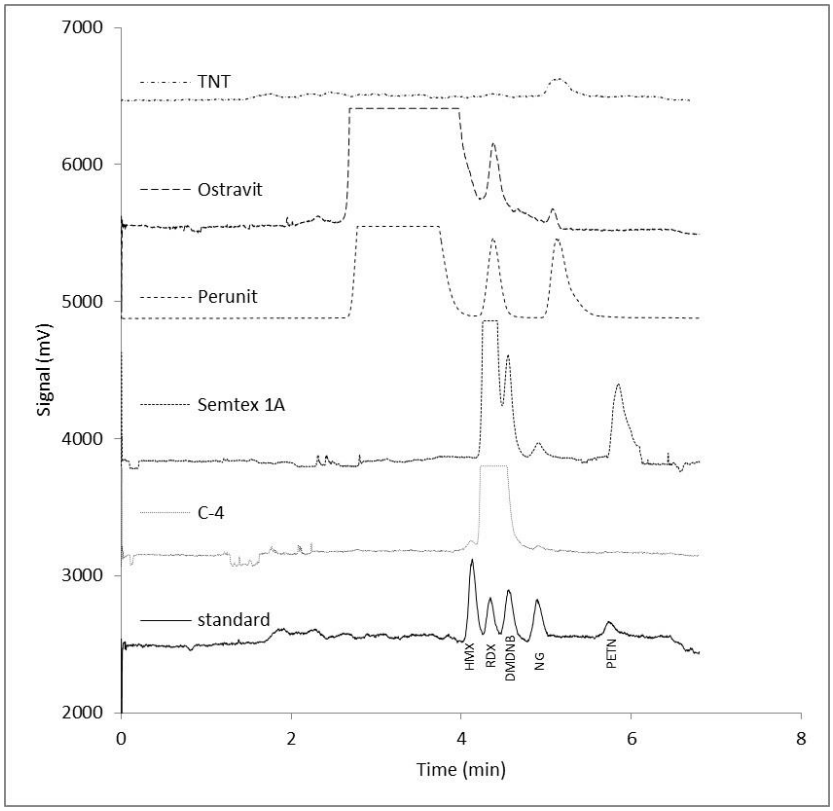

Fig. 2. Chromatogram of civilian secondary explosives compared with standard mixture.

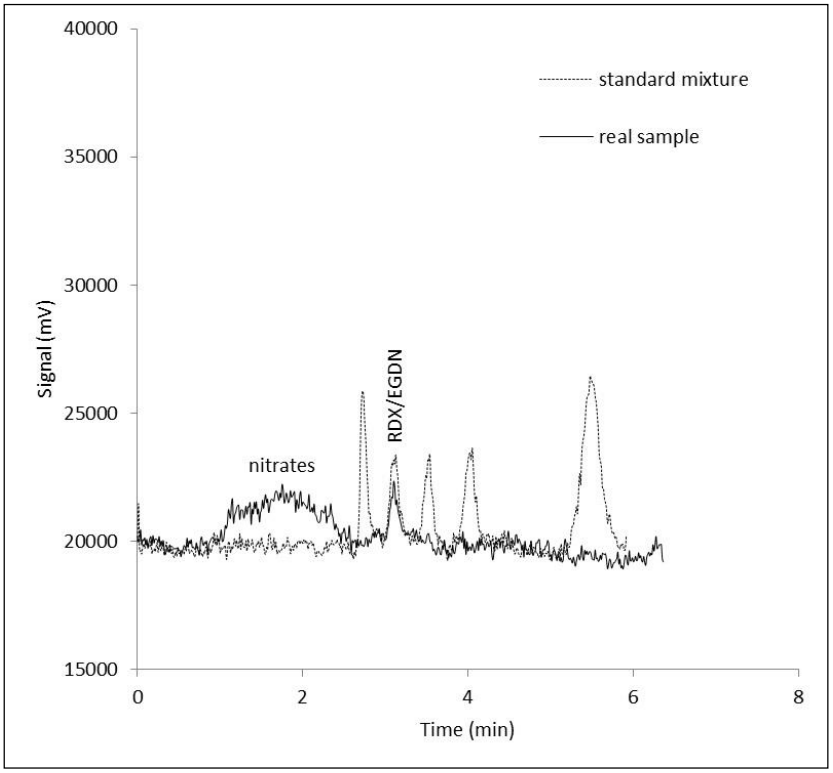

Fig. 3. Chromatogram of real sample soil compared with standard mixture.

4 Conclusions

The novel portable device for the analysis of energetic materials has been developed. The device consists of the miniaturized microcolumn liquid chromatograph, photolytic converter and the chemiluminescence detector with top selectivity for determination nitramine-, nitroester- and most of nitroaromates-based explosives. Proper function of the device was demonstrated by analyses of standards, spiked sample of soil, and finally real samples of soil from ammunition disposal place. The developed device is suitable to provide fast and very selective determination of nitrates and organic nitramines and nitroesters at trace concentrations without the need of complicated sample pre-treatment. Resistance to the contamination and to long term decommissioning is another benefit of the designed novel system. The dimensions and integrated power supply predestine the device to be used in terrain.

Acknowledgement

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P6 DETERMINATION OF BIOCHEMICALLY IMPORTANT FLAVINS USING CAPILLARY ELECTROPHORESIS WITH LASER INDUCED FLUORESCENCE DETECTION

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Summary
Riboflavin, known as vitamin B_2, is a precursor of important redox cofactors - flavin mononucleotide and flavin adenine dinucleotide. Its basic structure contains isoalloxazine consisting of three condensed heterocycles. Rigidity of this structure is responsible for native fluorescence of flavins and therefore can be easily and sensitively detected using fluorescence detection without derivatization. Optimization of separation method based on capillary electrophoresis coupled with laser induced fluorescence detection for determination of riboflavin in urine and beverages was performed.

1 Introduction

Cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) are biochemically important flavins, which are prosthetic group of many enzymes involved in oxidative phosphorylation, respiratory chain and metabolism of xenobiotic. Precursor of FMN and FAD is riboflavin, known as vitamin B_2. Riboflavin is water-soluble vitamin that is essential for higher organisms. Riboflavin is not stored in human body and is readily excreted in urine. The basic structure contains isoalloxazine consisting of three condensed heterocycles. Rigidity of this structure is responsible for native fluorescence of flavins and therefore can be easily and sensitively detected using fluorescence detection without derivatization. Optimized separation method based on capillary electrophoresis coupled with laser induced fluorescence detection (CE-LIF) was used for determination of riboflavin in urine before and in two-hour intervals after taking one pill of B-Komplex (Zentiva, Prague, Czech Republic) and in beverages such as alcoholic and nonalcoholic beers, beer lemonade, energy drink and fruit juice.

2 Experimental

2.1 Samples

Stock solution of 10 mM riboflavin was dissolved in 0.1 M NaOH. Stock solutions of 10 mM FMN and FAD were dissolved in deionized water. Model sample used for optimization of separation conditions was composed of 1 µM riboflavin, FMN and FAD diluted from stock solutions by deionized water. Samples of urine and beverages were centrifuged 5 minutes at 10 000×g and diluted in deionized water. For determination of creatinine, urine samples were diluted in 66 % (v/v) acetonitrile in

deionized water and centrifuged 10 minutes at 10 000×g due to precipitation of proteins.

2.2 Method

The Agilent G7100A CE System (Agilent Technologies, Waldbronn, Germany) coupled with external ZETALIF™ LED 480 detector (Picometrics, Toulouse, France) λ_{ex} 480 nm was utilized in this study. The photomultiplier tube (PMT) voltage was 600 V and rise time (RT) was set to 0.5 second. Separation conditions were based on the article published by *Hühner et al.* [1] and were further optimized. Background electrolyte (BGE) was composed of 50 mM sodium tetraborate, pH 9.7, pH was adjusted by 1 M NaOH. BGE was filtered using nylon membrane filter with 0.45 μm porosity and degassed in an ultrasonic bath for 10 minutes. Fused-silica capillary of 61.0 cm total length (L), 40.0 cm effective length (L_{ef}) and 50/375 μm inner/outer diameters (ID/OD) was used. Capillary was kept at 25 °C and separation voltage was 30 kV. Sample diluted in deionized water was introduced into the capillary using pressure of 50 mbar for 6 seconds. The determination of creatinine in urine to normalize its excretion volume was performed by previously described method based on capillary electrophoresis equipped with capacitively coupled contactless conductivity detection (CE-C^4D) using BGE consisted of 8 % (v/v) acetic acid and 0.1 % (w/w) (hydroxyethyl)-cellulose [2]. Fused-silica capillary of L 80.0 cm, L_{ef} 64.6 cm and 50/375 μm ID/OD was used. Capillary was kept at 30 °C and separation voltage was 30 kV. Urine sample diluted in 66 % (v/v) acetonitrile in deionized water was introduced into the capillary by pressure of 50 mbar for 12 seconds.

3 Results and Discussion

Electropherogram of the separation of model sample consisting of 1 μM riboflavin, FMN and FAD under optimized condition is shown in Fig. 1. Calibration curves for determination of riboflavin using LIF detection in linear range 5-1000 nM and creatinine using C^4D in linear range 10-110 μM were measured. Limits of detection (LODs) were calculated according to signal-to-noise ratio (S/N) criterion, where S/N = 3, limits of quantification (LOQs) were calculated as S/N = 10. Obtained calibration curve equation for peak areas of riboflavin was $y = 3.09 \times 10^{-3} c_r + 2.36 \times 10^{-2}$, $[c_r]$ = nM. Calculated LOD and LOQ for riboflavin was 2 and 5 nM. Calibration curve equation for peak areas of creatinine was $y = 1.3 \times 10^{-2}\ c_c + 8.56 \times 10^{-3,}$ $[c_c]$ = μM. LOD and LOQ for creatinine was 1.9 and 6.4 μM.

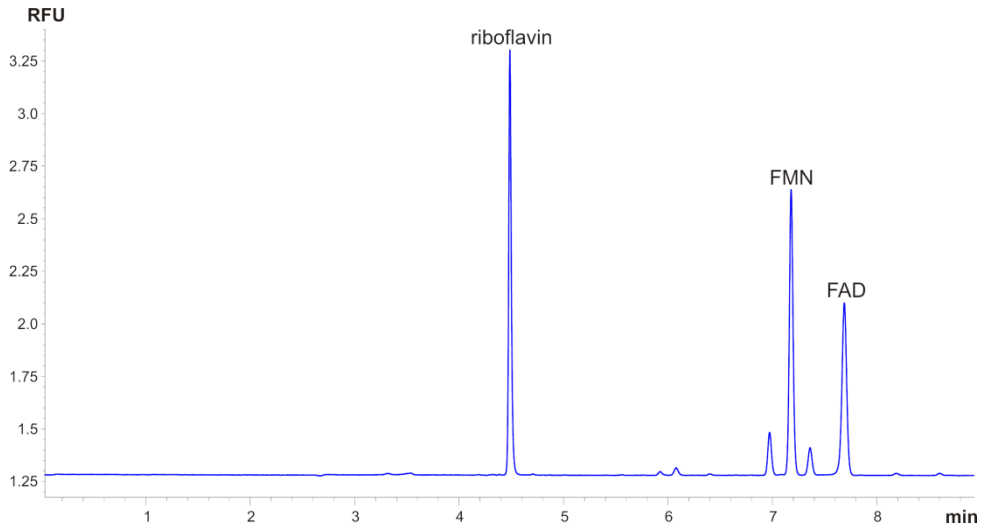

Fig. 1. Electropherogram of the separation of model sample consisting of 1 µM riboflavin, FMN and FAD under optimized conditions. BGE: 50 mM sodium tetraborate, pH 9.7; capillary: 50/375 µm ID/OD, 61/40 cm L/L_{ef}; voltage: 30 kV; temperature: 25 °C; injection: 50 mbar 6 s; detector: ZETALIF™ LED 480, λ_{ex} 480 nm, PMT 600 V, RT 0.5 second.

Samples of urine and beverages were treated as described above and analyzed three times. Concentration of riboflavin in urine samples was related to the excretion of creatinine. Kinetics of riboflavin excretion in the urine in two-hour intervals after taking one pill of B-Komplex is shown in Fig. 2. As seen, the most of unused riboflavin is excreted in urine in 6 hours.

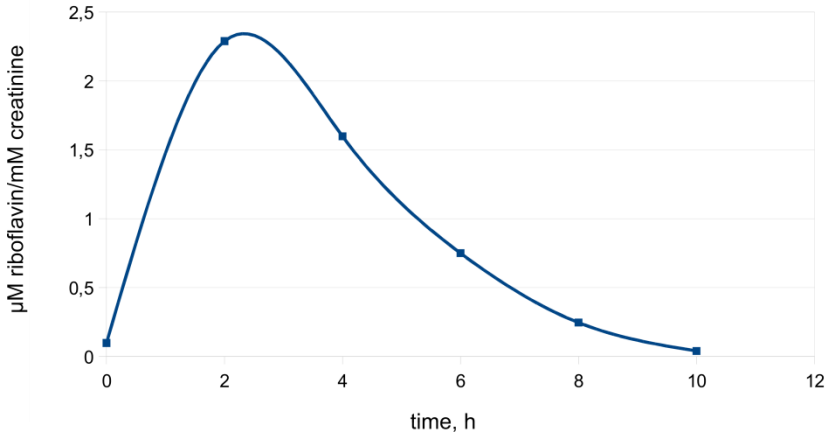

Fig. 2. Kinetics of riboflavin excretion in the urine in two-hour intervals after taking one pill of B-Komplex.

The lowest concentrations of riboflavin in beverages were measured in beer lemonade and nonalcoholic beer. As expected, the highest concentrations were measured in fruit juice and energy drink, probably because riboflavin is widely used in the food industry as a natural colorant (designation code E 101).

4 Conclusions

In this study, method optimization for determination of riboflavin, FMN and FAD was performed. Optimized method was used for determination of riboflavin in urine and beverages such as alcoholic and nonalcoholic beers, beer lemonade, energy drink and fruit juice. The most of unused riboflavin is excreted in urine in 6 hours after taking one pill of B-Komplex. The lowest concentrations of riboflavin in beverages were measured in beer lemonade and nonalcoholic beer. The highest concentrations were measured in fruit juice and energy drink.

Acknowledgement

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P7 ELUCIDATING PROTEIN POSTTRANSLATIONAL MODIFICATIONS USING COMBINATION OF RECOMBINANT PROTEIN SPECTRAL LIBRARY AND IN SILICO DESIGNED SRM ANALYSIS

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Summary

Posttranslational modifications (PTMs) of proteins represent fascinating extensions of the dynamic complexity of living cells' proteomes, but also present a solid obstacle in the proteome analysis. Identification and mapping of PTMs in proteins have improved dramatically, but to comprehend complex mechanisms and biological functions, one must address also very low abundant proteins. Here, we demonstrate *in silico* derived analysis of a low abundant target of ubiquitination and the MS/MS identification of the predicted ubiquitination sites.

1 Introduction

The protein PTM analysis is a challenging task that requires an advanced methodology [1] and usually also a deal of luck. Here, we employed *in silico* analysis and state-of-the-art mass spectrometry to determine position of a known PTM. Our model is a 60 kDa protein that is regulated by proteasome mediated degradation pathway.

2 Experimental

Protein of interest was extracted by immunoprecipitation from transgenic *Arabidopsis* line bearing its fusion with GFP under *35S* promoter. In parallel, recombinant protein (native sequence) was expressed and purified from *E. coli*. Protein ubiquitination *in vivo* was confirmed by Western blot analysis. Material for LC-MS analysis was prepared as described previously [2]. The resulting peptides were then analyzed online by nanoflow C18 reverse-phase liquid chromatography using a Dionex Ultimate 3000 RSLC nano UPLC system (Thermo) directly coupled to a nanoESI (electrospray ionization) source CaptiveSpray (Bruker) and an UHR maXis impact q-TOF mass spectrometer (Bruker) [3, 4], or TSQ Quantiva triple quadrupole (Thermo). The SRM method was designed by Skyline 3.1 (MacCossLab Software; https://skyline.gs.washington.edu).

3 Results and Discussion

Protein ubiquitination is an important PTM in plant hormone signaling and thus the sites of ubiquitination are interesting candidates for selective mutation. The sequence of our protein of interest contains our 20 lysine residues and it is not clear which are involved in its regulation. Its amount *in planta* is relatively low, but by employing a 35S overexpressor and immunoprecipitaion, we were able to detect its ubiquitination (Fig. 1).

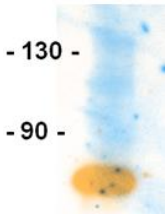

Fig. 1. Western blot validation of protein ubiquitination *in vivo*. Overlay of a consecutive staining by anti-GFP (orange) and anti-ubiquitin (blue), the relative molecular mass is indicated.

However, the protein amount for an untargeted LC-MS was not sufficient to elucidate the PTM position (the protein coverage was below 10%). We have prepared a recombinant version of our protein and the resulting MS/MS tryptic peptide library covers 78% of the protein sequence. The remaining 22% cannot be reached by a

trypsin digestion. We tested our library and 42 peptides based on SRM designed and optimized for the recombinant protein could be traced in immunoprecipitated samples, though the intensities of some targets indicated a presence of an additional PTM. As the next step, we designed SRM for predicted ubiquitination sites. Altogether, SRM for over 1,400 peptides were tested. The analysis pointed out four ubiquitination sites. To provide further evidence, we used TUBE1-agarose matrix (Tandem Ubiquitin Binding Entities) to enrich ubiquitinated proteins from plant extracts and we were able to detect at least one of the determined ubiquitination sites (Fig. 2).

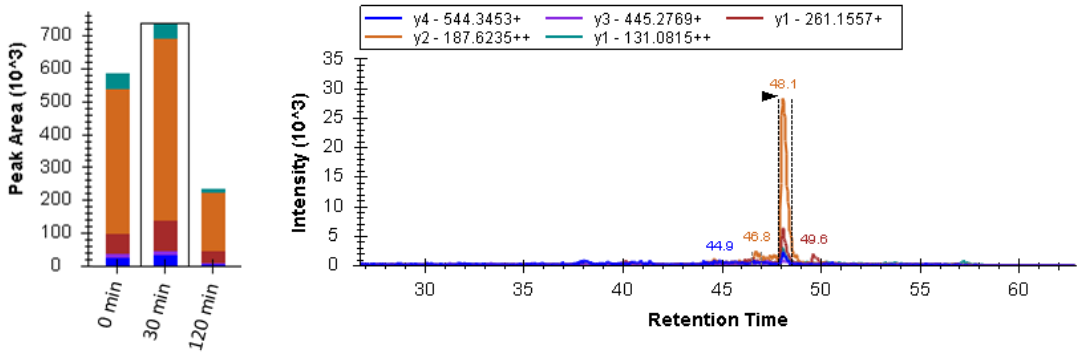

Fig. 2. Targeted analysis of identified ubiquitination site in enriched plant protein extracts. The bar plot indicates induced protein degradation (samples were harvested 30 to 120 min after the induction of degradation).

4 Conclusions

In conclusion, we were able to pinpoint *in vivo* ubiquitinated peptides of a low abundant protein. This study demonstrates the potential of modern technology and we believe that this approach could be the next level in elucidating complex PTMs in cell signaling pathways.

Acknowledgement

This work was supported by the Czech Science Foundation [P305/12/2144] and European Regional Development Fund for 'CEITEC–Central European Institute of Technology' [CZ.1.05/1.1.00/02.0068].

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P8 DEVELOPMENT OF PROTOCOLS FOR PROCESSING OF TWO- AND THREE-DIMENSIONAL SEPARATION DATA

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Summary
In this work, the algorithms for complex evaluation of two- and three-dimensional data were developed and tested on LCxLC and LC-CE separations of biologically active compounds. The data processing included unequivocal identification of compounds, visualization and quantification procedures.

1 Introduction
Two-dimensional liquid-phase separation techniques are becoming routinely used in analytical laboratories for characterization of complex mixtures. Although there are general guidelines available in the literature for optimization of LCxLC separations [1], the processing of collected data is still a difficult task, especially when multichannel detection is used and precise quantification is required [2].

2 Experimental
The experimental data used in the study were obtained using two-dimensional LCxLC, LC-MEKC and LC-CZE analyses of biologically active compounds as described elsewhere [3]. The data collected from both, UV detector and reconstructed chromatograms obtained using mass spectrometer were exported in the ASCII format, converted to the matrix with rows corresponding to the fraction cycle periods using proprietary synchronization software. The developed algorithms were programmed in Python object-oriented language (version 3.3.5.) with Matplotlib plotting library (version 1.3.1.).

3 Results and Discussion
Data processing is an integral part of development of multidimensional separation methods. This work was focused on development of algorithms enabling pre-processing of the data, i.e. background subtraction, data smoothing, correction of minor shifts due to the application of programmed working conditions [4,5] and deconvolution of co-eluting compounds. The procedures for quantification based on integration of primary data rather than final two-dimensional chromatograms have

been developed. The method for construction of three-dimensional chromatograms is proposed and tested on comparison of the separations in orthogonal modes of hydrophilic interaction liquid chromatography with reversed-phase liquid chromatography and also with electrophoretic separations (zone electrophoresis, micellar electrokinetic chromatography). The algorithm based on peak deconvolution and visualization of peaks as ellipsoids, taking into account intensity of recorded signal and peak widths at certain heights in all three dimensions, is suitable for complex evaluation of peak capacity and orthogonality of separation achievable by three-dimensional combination of separation methods.

4 Conclusions

In this work, the algorithms for processing of two- and three-dimensional data were developed for both, identification of separated compounds as well as their quantification. The procedures can be widely applied for characterization of complex mixture separations.

Acknowledgement

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P9 STUDY OF THE CELL WALL OF *STAPHYLOCOCCUS AUREUS* AND ITS SENSITIVITY TO ENZYBIOTICS

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Summary

The endolysin resistant and sensitive strains of *Staphylococcus aureus* were compared by means of structural analysis of peptidoglycan isolated from their cell walls.

Purified peptidoglycan samples were treated with lytic enzymes and obtained specific fragments (peptides, muropeptides, oligosaccharides) were analyzed by the developed method using high-performance liquid chromatography with mass spectrometry detection (HPLC-MS). The structural explanation of the resistance of the studied strains was suggested.

1 Introduction

The increasing resistance of pathogenic microorganisms to many antibiotic preparations leads to a necessity to find new types of antimicrobial agents. A promising alternative to antibiotic therapy is the use of "enzybiotics", i.e. bacteriophage lytic enzymes (endolysins), which are able to specifically hydrolyze the cell wall components and thereby cause lysis of bacterial cell [1,2]. The efficiency of the recombinant lytic enzyme against *S. aureus* strains is almost complete, but there were found individuals, which are resistant to lytic enzyme treatment.

A key component of microbial cell wall contributing to its rigidity and stability are peptidoglycans. In peptidoglycan, a cross-linked polymer composed of glycan backbone branched with peptide stems. The peptide side chains are joined together directly or via peptide bridges. Whereas the saccharide part shows only low diversity, the peptide part can vary significantly in the composition of amino acids, sequence, and cross-linking. The basic structure of peptidoglycan is a characteristic of bacterial specie, although it should reflect changes in bacterial metabolism and life conditions. In the case of *S. aureus* peptide stems are L-Ala-γ-D-Gln-L-Lys-D-Ala(-D-Ala), the cross-linkage is usually built up from five or six residues of Gly [3]. The molecular principle of the resistance to lytic enzyme has been elucidated as changes in peptide cross-linkage, such as truncations [4].

2 Experimental

Peptidoglycan samples were isolated from dead *S. aureus* cells using modified purification protocol for removing undesirable biopolymers. Briefly, bacterial cells were repeatedly boiled in 4% solution of sodium dodecyl sulfate, collected cell walls were washed with hot water, treated with 50% hydrofluoric acid, and washed with water again [5]. Purified peptidoglycan was dissolved in PBS buffer and lytic enzyme rLys812F1 was added to the solution. After overnight digestion, the suspension was separated by centrifugation and the supernatant was analyzed by LC-MS technique without further treatment.

HPLC-MS experiments were focused on the separation and identification of individual products of enzymatic digestion of peptidoglycan. They were performed both on octadecyl (Poroshell 300SB-C18; Agilent) and porous graphite (Hypercarb; Thermo Scientific) columns. Chromatographic analyses were carried out with 1100 series (Agilent) instrument coupled with diode-array detector (Agilent) and ion-trap mass spectrometer amaZon equipped with an ESI ion source (Bruker Daltonics).
Structural analyses were based on MS and MS^n data obtained in both ion modes.

3 Results and Discussion

To optimize methodic procedure, time-consuming preparation of purified peptidoglycan was simplified by the elimination of non-essential steps with the preservation of efficiency and reproducibility. The selection of lytic enzyme was focused on the enzyme with narrow structural specificity and limited places of action, such as endolysin rLys812F1.

The method of choice for the description of peptidoglycan lysis by enzyme treatment was the combination of advanced analytical techniques, such as reverse-phase HPLC and MS. The porous graphite column showed better resolution of structurally closed species with the potential to semi-preparative application. The satisfactory separation of peptidoglycan fragments according to size and structure was obtained using methanol/water gradient (up to 30 % of organic solvent) with the addition of formic acid (0.1 %). The retention of target molecules is substantially increased by the presence of acid. However, the application of the hilic phase columns failed.

The structural study was allowed by electrospray ion-trap MS using the potential of multistage fragmentation experiments. The detected products of enzymatic treatment provided particularly deprotonated molecules ($[M–H]^-$) or sodium adducts ($[M+Na]^+$) according to the ion mode. In the case of resistant strains, the additional ions at 30 u above the sensitive ones were observed (Fig. 1.). Subsequent MS/MS experiments supported the idea that the difference was caused by presence of different amino acid residue.

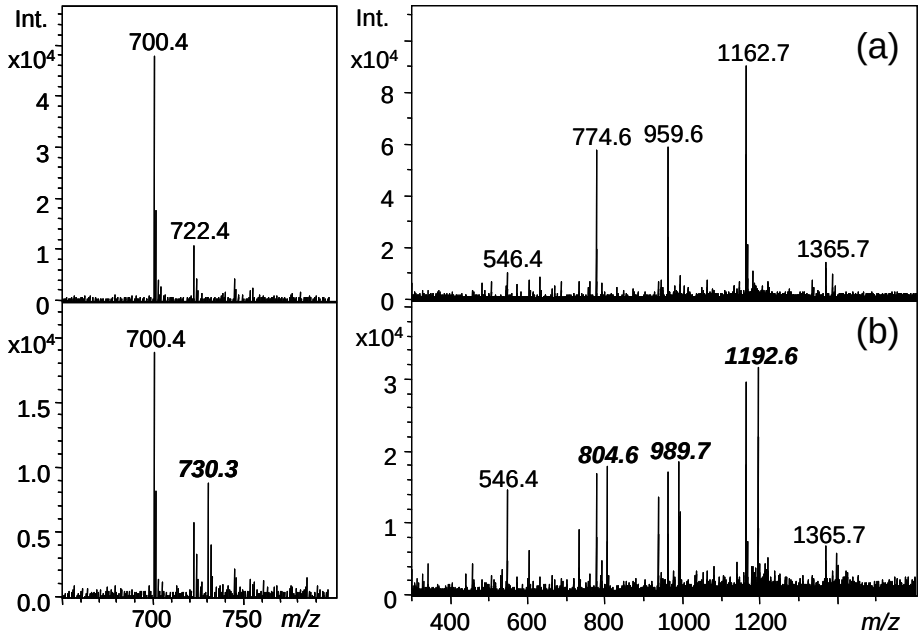

Fig. 1. MS spectra of the peaks corresponding to peptides derived from sensitive (a) and resistant (b) *S. aureus* strains peptidoglycan. Resistant-specific ions are labeled with bold italics.

4 Conclusions

The results showed the abundant species were detected and identified as peptides and muropeptides derived from peptidoglycan. The inter-strain differences in the peptidoglycan structure of *S. aureus* were elucidated after the action of lytic enzymes. LC-MS experiments, especially MS/MS of selected peptidoglycan fragments indicated, that resistance of bacterial strain is connected to the changes in amino acid sequence in peptide bridge – substitution of one Gly residue with Ser one.

Acknowledgement

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P10 METAL CONCENTRATIONS IN URBAN AEROSOL IN BRNO AND IN EXHAUST FUMES

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Summary

Total metal concentrations in aerosol samples and exhaust fumes as well as the bioaccessibility of metals in simulated lung fluids were determined. The extraction *in vitro* seems not to be dependent on the surface tension of extraction solution.

1 Introduction

Increasing environmental burden in urban agglomerations including metals from fuels combustion, traffic and industry, poses considerable health risks. The aim of this

research was to evaluate the bioaccessibility of selected metals contained in urban aerosol and in exhaust fumes.

2 Experimental

An urban aerosol (PM1) in Brno and exhaust fumes from various fuels were sampled. To assess the degree of potential intoxication by inhalation way, the extraction in solutions of simulated lung fluids was chosen. Gamble's solution (extractant A) was suggested as simulated interstitial fluid for in-vitro testing [1]. Artificial alveolar fluid (B) is the Gamble's solution with 1,2-Dipalmitoyl-sn-Glycero-3-Phosphatidylcholine (DPPC). Artificial lysosomal fluid (C) simulates a function of alveolar and interstitial macrophages [2]. Simulated lung fluid based on DPPC was newly proposed (D). (DPPC is the main substance for surface tension reducing [3].) The saline (E) and deionized water (F) were also tested. The determination of total metal contents and their bioaccessible proportions was performed by ICP-MS. The surface tension of simulated lung fluids was observed too.

3 Results and Discussion

The contents of Al, Cd, Ce, Cr, Cu, Fe, Mn, Ni, Pb, V, and Zn in samples were determined. Generally, total contents of metals in exhaust were low, thus, the bioaccesssible fractions were oftentimes under the limits of quantification. Simulated lung fluids evinced significantly different extraction efficiency for metals in urban aerosol samples. A higher ionic strength of extraction solution or a lower surface tension of the solution had no clear effect on increase in extraction efficiency.

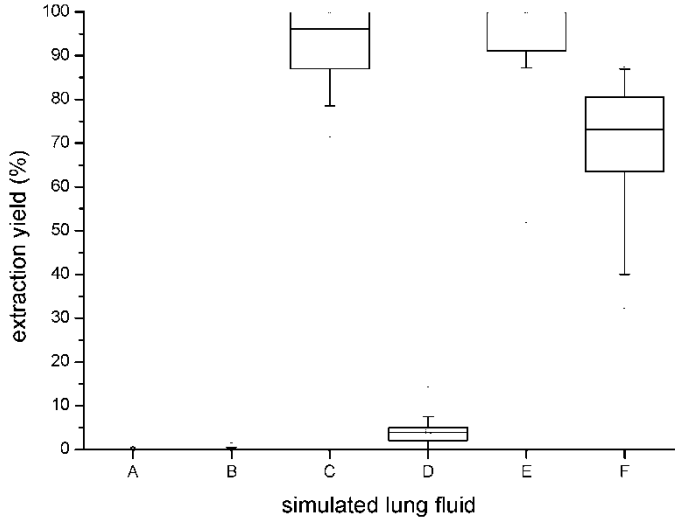

Fig. 1. Bioaccessibility of Cd in simulated lung fluids.

4 Conclusions

Traditionally used Gamble's solution evinced the lowest extraction power, which could lead to a significant underestimation of the bioaccessibility of metals in aerosol. A high metal solubility in artificial lysosomal fluid is probably caused by acidic pH.

Simulated lung fluid based on DPPC had lower extraction efficiency than deionized water despite lower surface tension.

Acknowledgement
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P11 SYNTHESIS AND ANALYSIS OF QUANTUM DOT CONJUGATES INTENDED FOR FRET SENSOR

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Summary
Our work is focused on synthesis of sensor based on Förster resonance energy transfer (FRET), which is aimed to be used for DNA mutation detection. The sensor is based on the attachment of laboratory synthesized quantum dot (QD) to ssDNA. A complementary chain of a sample is labeled by a fluorescent dye. Thus, only if dsDNA between both complementary chains is formed, the energy from QD (donor) can be transferred to the dye (acceptor) and FRET is observed.

1 Introduction
Quantum dots provide many advantages, e.g. wide range of excitation wavelengths, size- and composition-tunable emissions, narrow and symmetric emission spectra, good quantum yields, relatively long size dependent luminescence and low photobleaching [1]. Moreover, their high extinction coefficients make them ideal FRET donors for transfer of large amounts of energy.

2 Experimental

We synthesized CdTe QDs by a modified two-step reaction [2]. Formed nanocrystals of QDs were coated by 3-mercaptopropionic acid (MPA) to make them negatively charged and water soluble. Finally, the surface was modified by thiolated polyethylene glycol (PEG) to boost the stability of solution. We conducted the experiments simultaneously on commercial QDs and on laboratory synthetized QDs to compare their properties and stability. Laboratory made and commercial QDs were attached to ssDNA via several different conjugation procedures, e.g. biotine-streptavidine, exchange of ligands and zero-length crosslinker reaction [3]. The ssDNA-QD conjugate was exposed to a model complementary ssDNA labelled with fluorescent dye 6-carboxyrhodamine (ROX), which was purchased from Sigma-Aldrich, Germany. In proposed experiments QDs act as energy donors and ROX labels as acceptors. The hybridization was performed using a standard annealing protocol and yield of hybridization verified by fluorescence spectra measurements.

3 Results and Discussion

The coating of PEG was attached to QDs to improve the stability of laboratory prepared QDs. We used capillary electrophoresis with laser induced fluorescence detection (CE-LIF) and fluorescence spectroscopy for inspection of synthetized coated QDs. While a shift in migration times is evident in Fig. 1A, the intensity of fluorescence emission in Fig. 1B remained unchanged.

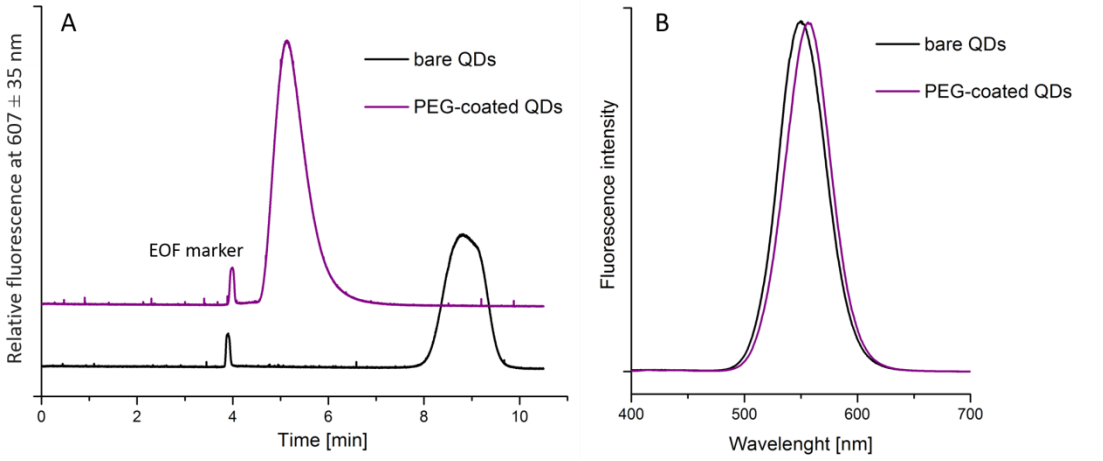

Fig. 1. A) CE-LIF of bare and PEG modified QDs. Uncoated capillary 30/40 cm, 50 mM TRIS/TAPS buffer, pH 8.6, voltage -10 kV, electrokinetic injection 5s. B) Fluorescence spectra of QDs after excitation at 340 nm.

CE-LIF and fluorescence spectroscopy were also used for monitoring of conjugation and hybridization reaction. After the exposure of ssDNA-QD conjugate to complementary ssDNA labeled by ROX, an increase in fluorescence intensity at

610 nm (the emission spectra maximum of ROX) is observed indicating a proper function of FRET sensor.

4 Conclusions

The fluorescence spectroscopy and CE-LIF was used for characterisation of QDs synthesis and modification as well as for proving successful conjugation between QD and ssDNA. Finally, the proper function of FRET-sensor was confirmed by the increased emission intensity of ROX after the hybridization with a complementary ssDNA.

Acknowledgement

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P12 NEW GENERATION OF DEEP-UV LEDS INCORPORATED IN PORTABLE ROBUST LOW COST DETECTORS FOR MICROFLUIDIC AND MINIATURISED ANALYSIS

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Summary

An effective use of optical detection, especially in microfluidics, aimed at portable, robust and relatively inexpensive platforms requiring inexpensive, commercially available, miniaturized, robust and analytically well performing light sources. The advantages of simplicity, low cost and efficiency is represented by detection systems

using light emitting diodes (LEDs) as light source with a special attention to recently developed new generation of deep-UV LEDs (below 300 nm). Previous generations of deep-UV LEDs had significant drawbacks such as low optical power (in the range of μW), strong parasitic emission in the visible spectrum, short lifetime and excessive heat generation .The new generation of deep-UV LEDs has significantly improved these characteristics. In this work we investigated optical performance of new generation of deep-UV LEDs with emission maxima at 255 and 280 nm. The optical power and emission spectra were tested. These LEDs were used in in-house photometric detector for use in a portable capillary FIA and LC system based on a microfluidic platform. The application of this detector was demonstrated for isocratic LC separation of parabens.

1 Introduction

Photometric detection in the deep-UV region (wavelengths bellow 300 nm) is of fundamental importance in detection and monitoring of numerous analytes in areas of (bio)chemical analysis, forensics, pharmaceutical, environment and other applications. Benefits of portability of analytical devices would be lost for systems dependent on bulky and heavy detection components. Especially in the area of optical detection, it is still a problem to source small and low cost but well performing commercial devices, compatible with miniaturized analytical apparatus. Recently new inexpensive, simple and robust deep UV LEDs became commercially available [1], which opened up new possibilities for design of portable absorption and fluorescence detectors for capillary flow-through techniques such as flow injection analysis (FIA), liquid chromatography (LC) and capillary electrophoresis (CE). The lowest wavelengths LEDs commercially available are at ~240 nm. This wavelength region below 250 nm has shown only a slowest progress. From the first use of a deep-UV LED (255 nm, only ca 15 μW) in an “in-house” on capillary detector in CE [2], next articles described to use the same kind of LED. These LED were very limited in their optical power, and had a very short lifetime. Recently a new generation of deep-UV LED based on different semiconductor materials emerged. This new generation of deep-UV LED has been demonstrated in a CE system and this work has been published this year [3]. In this work we present characterization test and use a newer line of LEDs, offering long lifetime and substantially higher optical power. Characterization of 255 nm and 280 nm deep-UV LEDs is detailed including a comparison with the older line deep-UV LED. Designs and applications are investigated, where those LEDs are incorporated into a simple low cost optical cell, in a high sensitivity Z-cell, or are combined with electrochemical detection. The detection designs are investigated with a modular flexible microfluidic platform used for miniaturized capillary LC [4].

2 Experimental

New generation of deep-UV LEDs based on AlN material with emission at 255 nm and 280 nm (OPTAN255H and OPTAN280J, Crystal IS, USA) was used. The obtained data were compared with the older generation of deep UV (UVTOP 255-HL,

SETi, USA). LED spectra were recorded using a fiber optics CCD Spectrophotometer (OceanOptics 2000, USA). For the determination of radiometric power a silicon photodiode (UV-100, Roithner-Laser, Germany) was used. The voltage and current were measured by a multimeter (Tenma, USA). A performance test of the deep-UV LEDs incorporated in an on-capillary detector was realized with the help of a capillary alignment interface (Agilent, Germany), and a silicone photodetector (TOCON_ABC2, SgLux, Germany) was placed on the opposite site of the capillary in an in-house designed holder [1]. The signal from the photodiode was connected to a data acquisition system (eDAQ, Australia), connected to a PC. This detector was then tested with a modular miniaturized LC station. The performance of this optical detection is demonstrated in an isocratic separation of a mixture of parabens [4]. Hyphenated detection technique with end-capillary screen printed electrode (Pt/AgCl) in wall-jet arrangement of electrochemistry cell (FC2-WJ, BVT, Czech Republic) was tested. Further, usage of the deep-UV LED combined with high sensitivity Z-cell (Knauer, Germany) was tested.

3 Results and Discussion

The previous generations of deep-UV LEDs, called here also older line, suffered drawbacks in terms of their low optical power (in the range of µW), strong parasitic emission in the visible spectrum range and broader bandwidth for major emission wavelength. The differences in emission spectra between the old and new lines of deep-UV LEDs are shown in Figure 1.

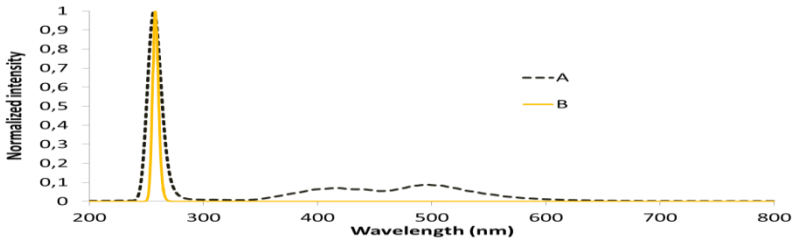

Fig.1. Comparison of old (A) and new (B) emission spectra of deep-UV LEDs.

Other problems for the previous old line of deep-UV LED was their short lifetime and excessive heating. Relatively recently marketed new generation of deep-UV LEDs exhibit significant improvements in those key parameters. In this work we investigated optical characteristics of representatives of this new line of deep-UV LEDs, specifically with emission maxima at 255 and 280 nm. We tested their optical power (0.6 and 1.5 mW, respectively) and emission spectra, showing a significant decrease in the visible parasitic emission bands. A summary of key parameters is shown in Table 1. The implementation of a deep UV absorbance detector into the miniaturised LC and FIA systems is shown. The short time baseline noise of was approximately 0.15 mAU, which is comparable with published literature. The hyphenation with electrochemistry end-capillary detection was successfully tested in

FIA mode and it is visualized by injection of 4 nL 1.1 mM methylparaben in water/MeCN 50/50. Finally, a combination of 250 nm deep-UV LED with a high sensitivity Z-cell is demonstrated showing a satisfactory performance.

Table 1. Selected parameters of older (UVTOP255-HL) and newer (OPTAN255H) line of deep UV LEDs.

Parameters	OPTAN255H	UVTOP255-HL
Radiometric power (mW)	0,57	0,014
Lifetime (Hours)	3000	200
Price (AUS $)	300	700
Emission band width (nm)	11	25
Compounds	AlN	AlGaN

4 Conclusions

New generation of LEDs was used for in-house designed on capillary photometric detector as a part of a portable capillary liquid chromatography system based on a microfluidic platform. The detector noise and sensitivity were satisfactory.

The advantage in the field of new technologies opened a way for next generation of low cost LEDs operating in the deep UV area and having a longer lifetime and sufficient optical power for analytical use.

Acknowledgement

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P13 SFC METHOD FOR THE ANALYSIS OF SYNTHETIC CANNABINOIDS AND THEIR METABOLITES

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Summary

Synthetic cannabinoids (SCs) are a group of structurally diverse compounds that were developed as pharmaceutical agents for the treatment of different diseases. However, they exhibit also the unwanted cannabis-like psychoactive effect [1]. This work is focused on development of SFC method for the screening analysis of some natural cannabinoids and a wide group of SCs and their metabolites in urine.

The optimized chromatographic system consisted of Zorbax Rx-SIL column and mobile phase composed of carbon dioxide/acetonitrile 93/7 (*v*/*v*). The other experimental conditions were: flow rate 2.5 mL/min, column temperature 40 °C, back pressure 95 bars and UV detection at 210 nm.

The developed analytical method was validated in terms of precision, selectivity, linearity, sensitivity, extraction recovery and robustness. The resulting relative standard deviations (RSD) for intra- and inter-day repeatability, related to retention time and peak area, were evaluated. The RSD values for retention were lower than 0.16% and 0.19% for intra-day and inter-day precision, respectively. Satisfactory results were also achieved for peak areas with RSD ≤ 5.12% and 6.58% for intra- and inter-day measurements. The limits of detection ranged from 0.12 to 0.52 µg/mL and limits of quantification from 0.40 to 1.73 µg/mL. The real sample preparation based on enzymatic hydrolysis and salting-out assisted liquid–liquid extraction was employed.

A rapid SFC method for simultaneous separation of the mixture of two natural cannabinoids, fifteen SCs and their metabolites in urine in a single run was developed and validated. The proposed method was proved to be suitable for screening toxicological analysis of important synthetic cannabinoids´ metabolites in human urine.

Acknowledgement

The authors gratefully acknowledge the financial support by the project NT13593-3/2012 from the Ministry of the Health of the Czech Republic, the project LO1305 of the Ministry of Education of the Czech Republic, the project 51021 of the Grant Agency of the Charles University in Prague and the project 364215 of the Grant Agency of the Charles University in Prague.

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P14 DETERMINATION OF BIOLOGICAL ACTIVE COMPOUNDS IN PRESSURIZED WATER EXTRACT OF *SAMBUCUS NIGRA* L. BRANCHES

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Summary
The determination of high-value substances in plant materials has been of increasing interest in last years. GC-MS and SDS-PAGE methods were employed for determination of carbohydrates and biological active proteins in pressurized hot water extract of *Sambucus nigra* L. branches.

1 Introduction

Sambucus nigra L. (commonly known as black elder) is a wide-branched shrubby tree growing up to 10 m. It grows in hedgerows, woods, coppices, and waste places throughout the Europe, Asia, North Africa, and the United States. All parts of the plant have been used for generations in folk medicine because of their essential positive effects on the human diet and health, which could be mainly ascribed to the presence of several taste and health related compounds such as sugars, amino acids, organic acids, polyphenols and also bioactive proteins [1].

The objective of this study was to determine sugars and bioactive proteins in pressurized hot water extract of *S. nigra* L. branches by GC-MS after derivatization and SDS-PAGE, respectively.

2 Experimental

2.1 Plant material

The braches of *S. nigra* L. growing in the Czech Republic were collected during autumn 2013, dried, ground to 6 mm size and stored in brown glass vials at laboratory temperature.

2.2 Extraction of *Sambucus nigra* L. branches

The pressurized hot water extraction (PHWE) was performed in laboratory-made extraction apparatus described before [2] at static arrangements. A portion (4 g) of the sample was put into 22 mL stainless steel extraction cell and extracted at a pressure of 15 MPa, temperature range from 60 °C to 160 °C with 20 °C steps, extraction time

1×5 min and nitrogen purge time 90 s after the extraction run. The resultant extract was cooled to 5 °C and stored in the fridge until GC or SDS-PAGE analysis.

2.3 GC-MS analysis

An aliquot of 1ml of the PHWE extract was lyophilized and derivatized by addition of 200 µl of BSTFA+TMCS (99:1, v/v), heated at 70 °C for 30 minutes, evaporated to dryness, dissolved in hexane and injected to the GC-MS system equipped with a DB-5MS capillary column (30 m × 0.25 mm i.d., polymer film thickness 0.25 µm, J&W Scientific, Folsom, CA) and PolarisQ mass spectrometer (Thermo Finnigan, San Jose, CA). The splitless injection technique was employed and the carrier gas was helium. Temperatures of the injector and the detector were 225 °C. The GC oven temperature was programmed from 50 °C to 300 °C. The mass spectrometer operated in a full scan mode in the range of m/z 50-650 and by electron impact ionization energy of 70 eV.

2.4 SDS-PAGE

Four hundred microliters of each water extract was mixed with 1.6 ml of ice-cold acetone. Samples were incubated overnight at -20 °C, and then centrifuged at 14,000 g for 10 min. The supernatant was carefully removed and the pellet was dissolved in 50 µL of the sample buffer (62.5 mM TRIS–HCl, pH 6.8, 2% SDS, 25% glycerol, 5% β-merkaptoethanol, 0.01% bromophenol blue). After brief boiling, 12 µl of each sample was applied onto the polyacrylamide gel (stacking gel 4% T, separating gel 12% T) Discontinuous gel electrophoresis was performed on Mini-PROTEAN 3 Cell (Bio-Rad, Hercules, CA, USA). Constant voltage of 160 V was applied to run the gel. Proteins visualization was carried out with CBB G-250.

3 Results and Discussion

GC–MS is excellent technique for the analysis of carbohydrates in plant samples; nevertheless the preparation of adequate derivatives is necessary. Derivatization reaction with BSTFA+TMCS is commonly used in GC-MS analysis of carbohydrates [3]. Chromatograms of carbohydrates standard mixture and PHWE extract of elder branches are presented in Fig. 1. Six carbohydrates, including xylitol, fructose, glucose, sorbitol, myo-inositol and sucrose, were identified in the sample by comparing retention times and mass spectra with those of authentic standards.

Protein content in elder branches was determined by SDS-PAGE. First the proteins were extracted by PHWE at different temperatures, precipitated with acetone and then analyzed by SDS-PAGE. The protein patterns of the respective extracts are shown in Fig. 2. These results indicate that the temperature 60 °C is the most suitable temperature for extraction of water-soluble proteins from elder branches. Higher temperature leads to protein degradation as can be seen in Fig. 2.

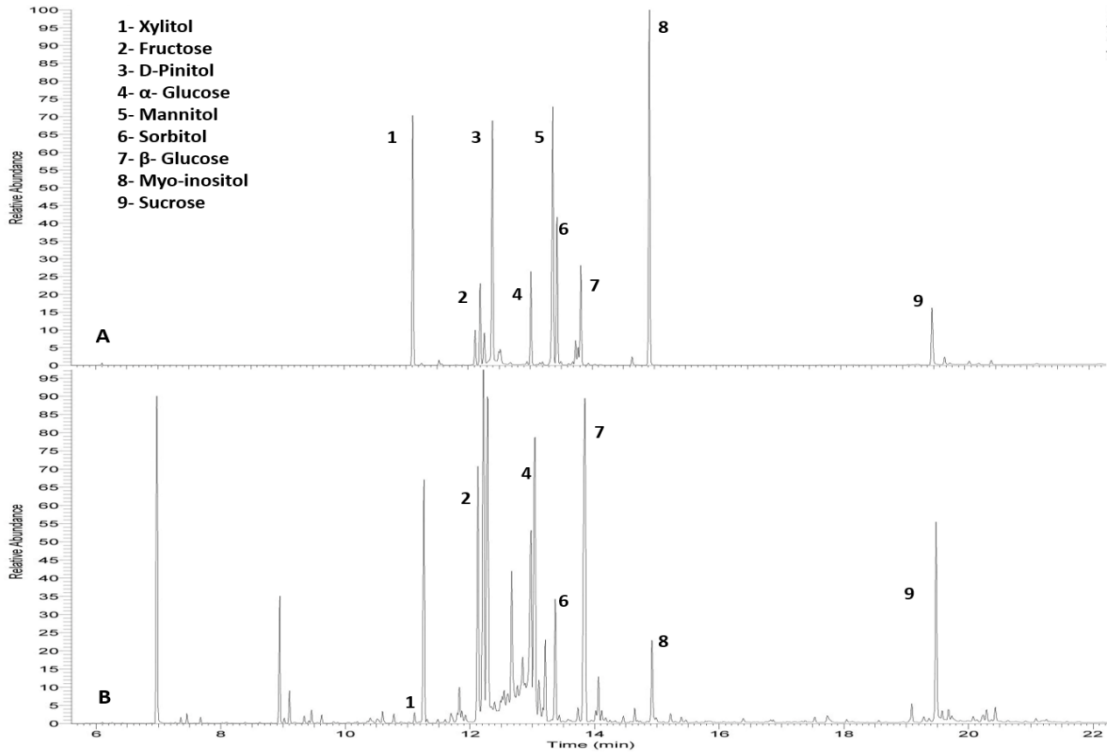

Fig. 1. GC-MS chromatogram of carbohydrates standard mixture (A) and PHWE extract of *S. nigra* L. branches (B).

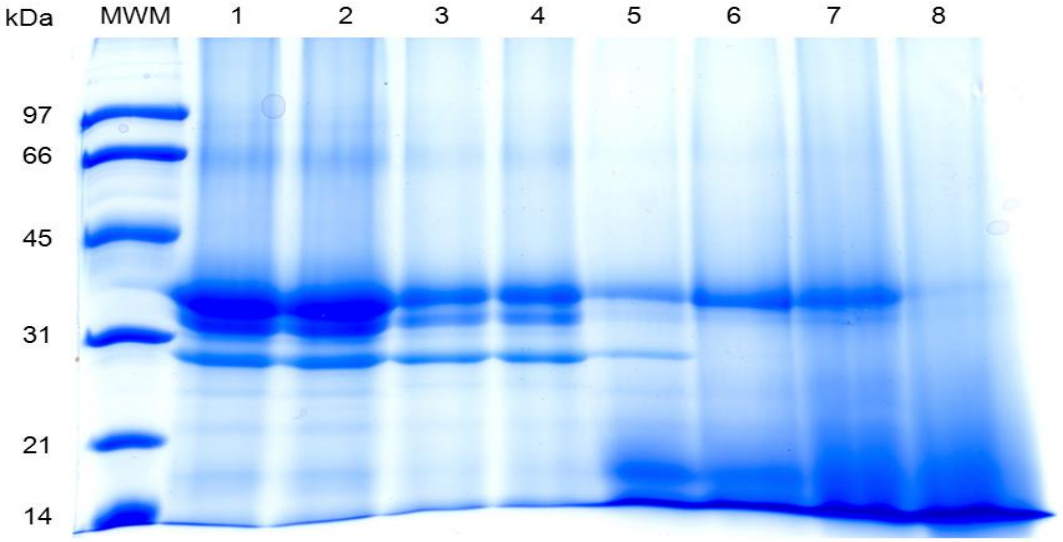

Fig. 2. SDS-PAGE of proteins extracted from elder branches by PHWE at different temperatures. MWM – molecular weight marker, 1,2 – 60 °C, 3,4 – 80 °C, 5 – 100 °C, 6 – 120 °C,7 – 140 °C, 8 – 160 °C.

4 Conclusions

The presence of carbohydrates and biological active proteins in pressurized water extract of elder branches by GC-MS after derivatization and SDS-PAGE was shown. The fructose, glucose and sucrose were determined as the most abundant carbohydrates. Moreover, the optimal conditions for extraction of water-soluble proteins from elder branches were found. The identification of these proteins and other valuable compounds will be the subject of our next research.

Acknowledgement

This work was supported by Regional Cooperation Project CAS (R200311521).

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P15 ANALYSIS OF ALLOXAN AND ITS PRECURSORS BY CAPILLARY ELECTROPHORESIS

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Summary

Alloxan is widely used agent for the induction of diabetes in experimental animal models of insulin-dependent diabetes. Because its use is prohibited in the European Union and because there is a hypothesis that alloxan is formed during the bleaching process of flour, it is of high interest to develop fast and reliable methods for determination of alloxan in food samples. In this work, we address this problem by developing a simple and fast capillary zone electrophoresis method for separation of alloxan, uric acid and barbituric acid. CZE analysis was achieved using optimized background electrolyte containing 50 mM sodium borate at pH 8.0. Within these conditions, all analytes were separated in two minutes with following migration order: uric acid, alloxan and barbituric acid. The method was then applied for analysis of three samples of bleached flour.

1 Introduction

In recent years, alloxan became one of the most popular diabetogenic chemicals in diabetes research. Alloxan is commonly used to induce diabetes in the lab animal models of insulin-dependent diabetes [1]. This fact was reported by Dunn and McLetchie as a result of the specific necrosis of the pancreatic beta cells [2]. The mechanism of alloxan action has been intensively studied, predominantly in vitro, and is now characterized quite well in many studies. Alloxan induces its diabetogenic action when it is administered parenterally, especially intravenously. In this case, the most frequently used dose of this drug to induce diabetes in rats is 65 mg/kg b.w**.** [3]. Alloxan initiates free superoxide radicals damage to DNA in pancreatic beta cells of the Langerhans islets, which are injured by necrosis leading to insulin deficiency and diabetes. As a glucose analogue, the agent accumulates in beta cells through uptake *via* the glucose trans-porter, GLUT2 [4]. This finding of "alloxan diabetes" has contributed significantly to understanding of pancreatic beta cell physiology and pathology. Alloxan-induced diabetes in animals shows similarities as diabetes mellitus in humans: body weight loss, polydipsia, polyuria, glycosuria, ketonuria, hyperglycaemia and ketonaemia [5].

For more than 10 years, alloxan is also discussed as an agent responsible for increasing of the number of patients with diabetes, mainly in the USA. The hypothesis says that alloxan is formed during the bleaching process of common flour. This process makes flour looking as "clean" and "beautiful". However, there is not any rigorous study to prove such hypothesis. Moreover, there are some unpublished studies with the information that those types of flour do not contain alloxan. Also the European Union prohibits using alloxan in any food stuff. These two facts (possible connection of alloxan, flour and diabetes and the EU interdiction) led us to the idea to develop a fast and simple analytical method to determine alloxan in food samples. Due to the structure of alloxan, we suggested two possible precursor of alloxan – uric acid and barbituric acid – that should be also covered in the analytical method.

As noted before, we focused on separation of alloxan and its precursors in this study. Since the method should be fast and simple, we decided to use capillary zone electrophoresis (CZE) which is very fast and user friendly. In our work, optimization of separation conditions, such as pH value and concentration of background electrolyte (BGE) was studied carefully. Finally, three bleached flours were analyzed by the developed method.

2 Experimental

2.1 Chemicals and reagents

Electrolyte components: borate acid and sodium hydroxide were purchased from Sigma Aldrich (St. Louis, MO, USA). The analytes standards: alloxan, uric acid and barbituric acid were bought also from Sigma Aldrich. Stock standard solutions of all analytes were prepared at a concentration of 1 mg/mL in deionized water (18 MΩcm, Millipore, MA, USA) and were appropriately diluted in deionized water for preparation working solutions at concentration of 50 μg/mL. All the chemicals were of analytical grade purity.

2.2 Apparatus

All the analyses were performed on the capillary electrophoresis system HP 3DCE (Agilent Technologies, Waldbronn, Germany) with the diode array detector. The UV detector was set at 254 nm. Uncoated fused silica capillaries (MicroSolv Technology, NJ, USA) with 50 μm i.d., total capillary length 33.0 cm, effective length 24.5 cm, hydrodynamic injection time of 5 s by 50 mbar and voltage of 20 kV were used in these experiments. The capillary cassette was thermostated at 25°C. The capillary was rinsed every day, before the initial experiment, with 0.1 mol/L NaOH (10 min), deionized water (20 min) and then with the running buffer (10 min). Before each analysis the capillary was rinsed with 0.1 mol/L NaOH (2 min), deionized water (3 min), and then with the buffer (5 min). All the rinsing was carried out under the pressure of 925 mbar. All of the measurements were performed three times unless stated otherwise.

3 Results and discussion

Alloxan, uric acid and barbituric acid are acidic compounds with negative charge under basic conditions. Hence, sodium borate background electrolyte at basic pH was chosen as a starting buffer for CZE experiments. Composition and pH was further optimized. First, we studied the effect of borate concentration in the range of 25 mM to 100 mM (keeping pH at 8.5). The optimal value was 50 mM. Lower concentrations resulted in broader peaks while higher concentrations produced irreproducible electric currents during analyses. Then, the effect of pH was tested. The pH of 8.0, 8.25, 8.5, 8.75, and 9.0 was studied. The best separation according to peak resolution and shape was achieved at the pH 8.0. The final conditions allowed separation of all the three compounds of interest within 2 minutes with following migration order: uric acid, alloxan and barbituric acid (Fig. 1).

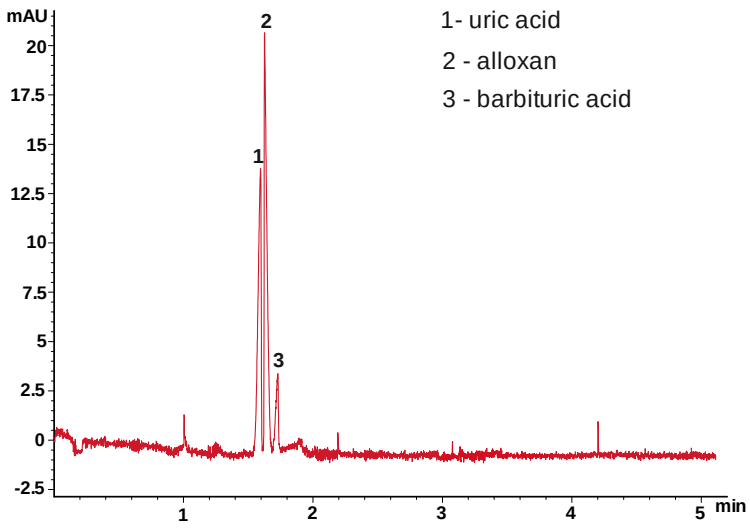

Fig. 1. Electropherogram of analyzed analytes. Operating conditions: 50 mM borate buffer, pH 8.0, 20 kV, 25 °C, injection 5 s by 50 mbar, sample concentrations: 0.05 mg/mL.

Then the method was applied for analysis of real samples of bleached flour. Three bleached flours from a common USA market – Pillsbury Best, Spartan, and Gold Medal – were analyzed. The samples were extracted 30 min by methanol using sonication bath. Then methanol was evaporated under stream of nitrogen and the samples were reconstituted in 10times diluted background electrolyte and loaded into capillary electrophoresis. All the samples analyzed did not contain alloxan, as well as uric acid and barbituric acid.

4 Conclusion

In our study, an easy and fast CZE method for separation alloxan and its precursors was developed and performed. The chosen method was applied to real samples of bleached flour and alloxan was not found in any sample. However, the CZE method can be used as a control method for food industry.

Acknowledgement

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P16 ADSORPTION BEHAVIOUR OF COPPER IONS ON ELDERBERRY, GOOSEBERRY AND PAPRIKA WASTE FROM AQUEOUS SOLUTIONS

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Summary

The adsorption of Cu(II) metal ions onto dried elderberry, gooseberry and paprika residues have been studied. The residues are generated in food industry and they may be potentially used for removing metal ions from wastewater. The fruits are a source of

biologically active compounds, such as flavonoids, phenols, carotenoids, capsaicinoids and vitamins, which exhibit various benefits to human health, but also sorption properties in relation to metal ions [1]. There are many methods to remove heavy metals from wastewater e.g. membrane separation, ion-exchange, coagulation, chemical precipitation and adsorption. However, biosorption is a promising technology to remove metal ions, because it uses cheap and environmentally-friendly biomaterials as agricultural by-products [2]. In this research the adsorption of Cu(II) metal ions from aqueous solutions at pH 4 has been investigated. The conducted experiments showed the effect of mass of different biosorbents on Cu(II) adsorption. The results suggest that elderberry, gooseberry and paprika waste may be an effective adsorbent for copper ions removal.

1 Introduction

Contamination of water by toxic substances and heavy metals from the industrial wastewater discharges is a growing worldwide environmental concern [3]. Heavy metals are very difficult to eliminate from environment even in small amounts. Therefore there is a need to seek and develop methods for metal ions removal [4-6]. Among many conventional methods one of the most promising technology is biosorption, which uses cheap and environmentally-friendly biomaterials [7]. The biomass is rich in dietary fiber and exhibits sorption properties in relation to metal ions. It is generated in food industry and its amount is estimated at about 10 – 35% by weight of the feedstock entering the production process. In Poland, the waste is generated up to 350 thousand tons per year and it has a tendency to grow. In recent years, there has been growing interest on the use of biosorption for the elimination of metal ions from industry wastewater. The elderberry, gooseberry and paprika biosorbents were selected to this research due to their promising potential for the removal of copper ions from aqueous media.

2. Experimental

Elderberry (*Sambucus nigra*), gooseberry (*Ribes uva-crispa*) and paprika (*Capsicum annuum*) waste were crushed into smaller particles, sieved and separated into fractions of different thickness. The biomass was dried to constant mass at a temperature of 60°C and then kept in a dessicator. The measurements were repeated three times. Deionized water was used to prepare all solutions. Adsorption experiments of Cu(II) ions were carried out by conventional batch wise method at room temperature. The pomace in the amount from 0.025 to 1.0 g and a portion of $CuCl_2$ test solution containing 10 mg/L of metal ions at pH 4 were placed in a conical flask and shaken in a shaker until equilibrium was reached. After that the contents were transferred into centrifuge tubes and centrifuged to separate phases for 15 min. at 4000 rpm. Then the solution concentrations of Cu(II) in portions above the biosorbent were measured by atomic absorption spectrophotometry (F-AAS) using SpectrAA 800 (Varian, Palo Alto, USA) apparatus at a wavelength λ = 324.8 nm for copper.

3 Results and Discussion

3.1 Effect of mass of biosorbent

The influence of mass of elderberry, gooseberry and paprika biosorbents on sorption efficiency for Cu(II) ions was investigated. Conducted experiments showed that the removal efficiency varied significantly with mass of residues. The percentage adsorption is demonstrated in Figure 1. It is observed that copper ions removal efficiency increases with increase in gooseberry mass, and decreases with increase in elderberry mass. Biosorption of paprika remained constant at about 41%. According to the results it is estimated that an optimum mass was reached at 0.5 g corresponding to 39.8% (paprika), 51.5% (gooseberry) and 60.9% (elderberry) removal and the maximum adsorption capacity of Cu(II) ions was indicated to be 2.18 mg/g, 1.43 mg/g and 3.40 mg/g, respectively (Figure 1, 2). The increase in adsorption efficiency may be attributed to greater number of sorption sites for the ions and high accessibility of metals to the binding sites with the mass increasing [8, 9].

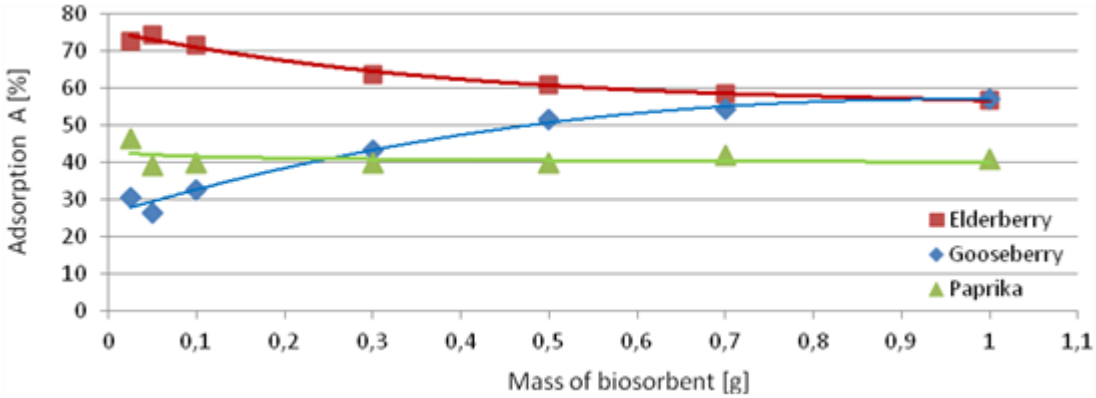

Fig. 1. The effect of mass of elderberry, gooseberry and paprika waste on adsorption *A* of Cu(II) ions (T = 25°C, C_0 = 11.72 mg/L, initial pH 4.02, particle size 0.212 mm, agitation speed 150 rpm, V = 10 mL, contact time 60 min).

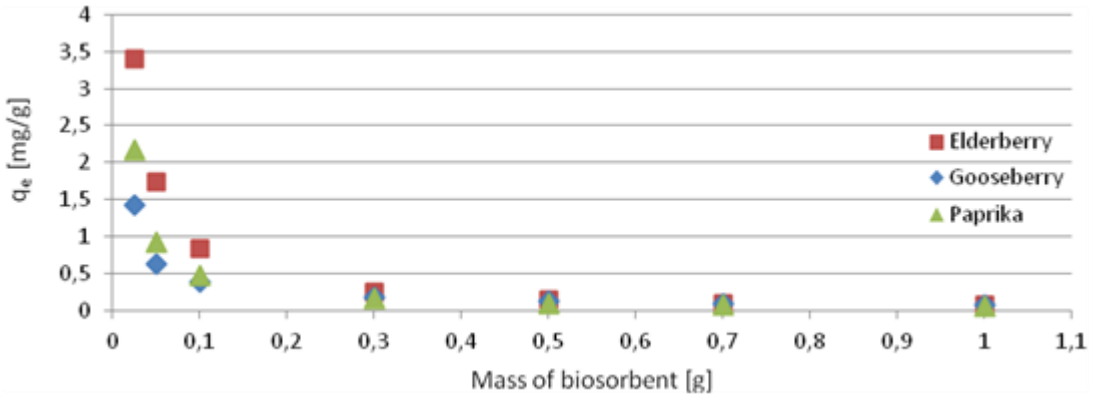

Fig. 2. The effect of mass of elderberry, gooseberry and paprika waste on q_e of Cu(II) ions (T = 25°C, C_0 = 11.72 mg/L, initial pH 4.02, particle size 0.212 mm, agitation speed 150 rpm, V = 10 mL, contact time 60 min).

3.2 Effect of the sorption of the metal ions on pH

The strong influence of the biosorption of copper(II) on pH was demonstrated in Figure 3. Initial pH 4.02 of solutions was decreased during the sorption process and it is dependent on the type of biosorbent and its composition. pH values have decreasing tendency with increasing mass of biosorbents. Solutions with paprika waste maintained constant pH of about 3.85. The gooseberry waste contributed to reducing values below pH 4 due to larger amount of compounds having an acidic group. Solutions with elderberry residues (0.025 g – 0.7 g), which were characterized by initial values the closest pH 4, caused the highest Cu(II) ions removal efficiency in comparison to other examined biosorbents.

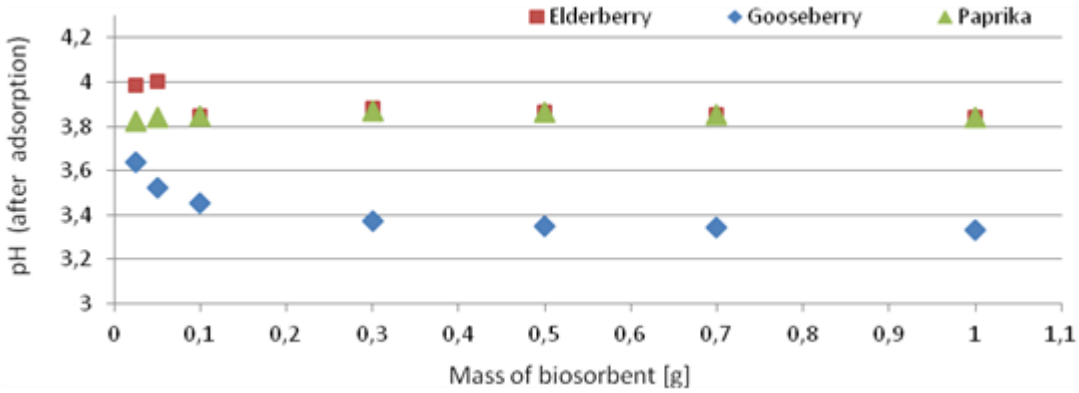

Fig. 3. The influence of the sorption of Cu(II) ions on pH values in aqueous solutions including elderberry, gooseberry and paprika waste (T = 25°C, C_0 = 11.72 mg/L, initial pH 4.02, V = 10 mL, particle size 0.212 mm, agitation speed 150 rpm, contact time 60 min).

4 Conclusions

Elderberry (*Sambucus nigra*), gooseberry (*Ribes uva-crispa*) and paprika (*Capsicum annuum*) residues are low-cost and readily available biosorbents for the removal of Cu(II) from aqueous solutions. The results showed that adsorption of Cu(II) ions depends on pH of solution and mass of the biosorbents. Among the analyzed sorbents the maximum adsorption of the metal ions was obtained on elderberry waste (A = 74.4%; q_e = 3.40 mg/g). The examined biomass has possibility to be used as effective adsorbent for copper ions removal.

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P17 SIMPLE FLOW-FOCUSING MICROFLUIDIC CHIP FOR DROPLET GENERATION

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Summary
There is an ascending requirement for analysis of chemical content of individual cells involving combination of various bioanalytical and optical sensors and arrangements. At the beginning of the cell analysis there is a need for creation of suitable transport vessels - droplets - that can be easily counted, sorted and serve for manipulation of cells with preserved biocompability as well. Among all of bioanalytical approaches droplet-based microfluidics proved its potential for producing droplets with precisely controlled size, shape as well as for encapsulation of reactants and delivering drugs. This study presents a simple microfluidic chip with flow-focusing geometry for generation of water droplets in fluorocarbon oil.

1 Introduction

Microfludics is a rapidly growing multidisciplinary field. Its advantages over conventional techniques include reduced reagent consumption, fast reaction time and short time of analysis. Droplet-based microfluidic devices based on water droplets in oil emulsion for encapsulating of living cells is a challenging field with wide range of potential practical applications. The research is still mainly focused on the technology, i.e., dynamic properties of droplets, stabilization of the interfaces and emulsions, formation of multiple core droplets, etc. [1]. Fluorocarbon oils with appropriate surfactants are essential for successful droplet generation/manipulation and their applications for the biochemical or biological assays [2, 3]. In this work we have optimized the droplet generator to be used for cell encapsulation.

2 Experimental

Simple design with flow-focusing geometry and two parallel droplet generators was selected (Fig.1). The width of the microfluidic channel was 60 μm and the height was 20 μm.

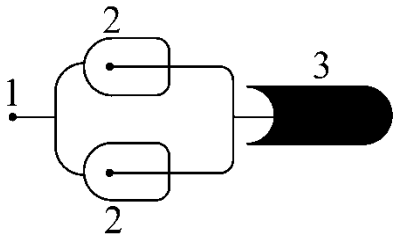

Fig. 1. Design of the microfluidic device: (1 - oil inlet, 2 - aqueous inlets (Methyl Green, Rhodamine), 3 - reservoir).

Microfluidic chip was fabricated using conventional lithography (Heidelberg μPG 101 Laser Writer) on Photomask Blanks (nanofilm, USA) followed by wet etching with hydrofluoric acid solution at 65 °C for 1.5 hour. Commercially available Sylgard 184 polydimethylsiloxane (PDMS) kit (Dow Corning, Midland, MI, USA) containing PDMS pre-polymer and cross-linker was used in the recommended ratio of 10/1 (w/w) [4]. Assembled chip was washed with 1% (w/w) trichloro(1*H*,1*H*,2*H*,2*H*-perfluorooctyl)silane solution. 3M Fluorinert™ FC-40 (Sigma Aldrich) with 1% (w/w) of Pico-Surf I (Dolomite Microfluidics, UK) was injected via the oil phase inlet and aqueous phases were injected via the aqueous inlets in the ratio oil/water of 6/1 (v/v). The images of water droplets were taken on a Fluorescence Lifetime Imaging Microscopy microscope (Intraco Micro spol. s r.o.) equipped with a MOTIC 5000 digital camera. For better visualization, Methyl Green and Rhodamine (Sigma Aldrich) were added into the water solutions.

3 Results and Discussion

Preliminary experiments were focused on the optimization of the conditions for droplet generation based on the data of Basova et al. [5]. Flow rate of 60 $\mu L \cdot h^{-1}$ for oil phase and 10 $\mu L \cdot h^{-1}$ for both water phases were set. Color droplets were easily recognized by the camera at the channel orifice (Fig. 2A).

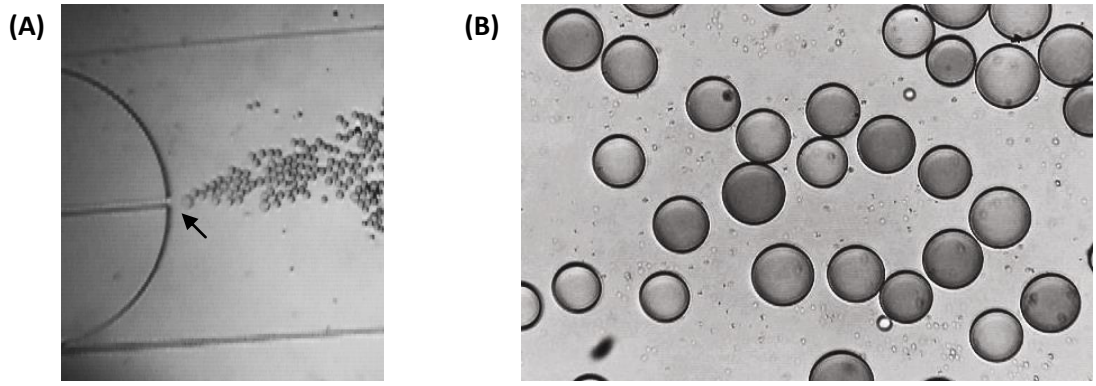

Fig. 2. Chip reservoir with orifice (A). Detail of Methyl Green (dark) and Rhodamine (light) droplets in the microchip reservoir (B).

Droplets were transferred via reservoir and collected in the Eppendorf tube. In order to stabilize the emulsion 1% (w/w) of commercially available surfactant in the FC-40 was used. The developed system was characterized by a stable water-in-oil emulsion and was stored under the mineral oil in the Eppendorf tube for a month. To monitor the synchronization of the droplet generation two parallel generators - aqueous inlets - (one with Methyl Green, second with Rhodamine) were used. As shown in Fig. 2(B) dyed droplets in the reservoir varied in their sizes (light vs. dark droplets). Observed non-uniformity in the size of the droplets indicates the presence of microgrooves in the microfluidic device. These microgrooves were formed during the procedure of etching with hydrofluoric acid solution [6]. To overcome this issue a lithography technique using spin-coating process with SU-8 photoresist followed by isotropic etching for smooth and well-defined channels should be applied for a replica master preparation.

Presented PDMS device with flow-focusing geometry can generate water-in-oil emulsion in a short time. It has been shown that a well-controlled production of monodisperse droplet emulsion will require better control of the fabrication (etching) protocol creating smooth channel surface.

Acknowledgement

Financial support from the Grant Agency of the Czech Republic (P206/12/G014) and the institutional support RVO 68081715 is acknowledged. We also thank to Central European Institute of Technology with research infrastructure supported by the project CZ.1.05/1.1.00/02.0068 financed by the European Regional Development Fund.

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P18 ORGANIC COMPOUNDS IN PM1 AEROSOL IN THE CENTRAL BOHEMIAN REGION IN THE CZECH REPUBLIC

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Summary
Contribution summarizes the concentrations of monosaccharide anhydrides, resin acids, methoxyphenols, monosaccharides, disaccharides, sugar alcohols, alkanes, hopanes, steranes and polyaromatic hydrocarbons in PM1 aerosols in small town Celakovice in winter. According to concentrations of organic markers, the identification of emission sources in Celakovice is discussed.

1 Introduction

Atmospheric particulate matter (PM) is known to play an important role in many environmental problems. During last years, much attention has been paid to the identification of emission sources of PM. To track the contributions of the main sources to composition of atmospheric aerosols, various source-specific organic tracers are analysed in collected PM [1-3].

2 Experimental

2.1 Aerosol sampling

Atmospheric aerosols in the size fraction PM1 were sampled over 12-h periods (daytime 6:00 – 18:00; nighttime 18:00 – 6:00) using a high-volume sampler (DHA-80. Digitel, 30 $m^3.h^{-1}$) on quartz filters during winter period of 2015 (21. 1. – 6. 2.) in small town Celakovice (12 thousand inhabitants) located about 9 km northeast from Prague (Prague-East District, Fig. 1).

2.2 Sample preparation, extraction and analysis

Collected aerosols were analysed for monosaccharide anhydrides (MAs), resin acids (RAs), methoxyphenols (MPs), monosaccharides (MSs), disaccharides (DSs), sugar alcohols (SAs), alkanes, hopanes, steranes and polyaromatic hydrocarbons (PAHs). Analysis of MAs, RAs, MPs, MSs, DSs and SAs included extraction of parts of filters with mixture dichloromethane/methanol (1:1 v/v) under ultrasonic agitation, derivatization of extracts with mixture of MSTFA + TMCS, dryness, redissolution in hexane and GC-MS analysis. Analysis of alkanes, hopanes, steranes and PAHs included extraction of parts of filters with mixture of dichlormethane/hexane (1:1 v/v), fractionation on column with silicagel, dryness to 1 mL and GC-MS analysis.

Fig. 1. Celakovice in the Czech Republic.

3 Results and Discussion

Analysed organic compounds had, in general, similar trend as the PM1 aerosols (Fig. 2). Concentrations of PM1 and analysed organic compounds were mostly higher during nighttime (18:00 – 6:00, Fig. 3) than during daytime (6:00 – 18:00). Presence of monosaccharide anhydrides in collected aerosols proves contribution of biomass burning to the composition of aerosols. Methoxyphenols, resin acids and retene indicate burning of lignin (i.e., wood), probably in the frame of residential heating. Presence of hopanes, steranes and phytane and pristane in aerosols collected in Celakovice proves contribution of emissions from traffic to aerosol composition. Steranes, phytane and pristane serve as specific markers of traffic while hopanes also serve as organic markers for fossil fuel combustion. Homohopane index [S/(S+R) = 0.08 (0.05 – 0.14)] and presence of picene indicate combustion of coal (brown coal) as the emission source of aerosols.

The average concentration of sum of PAHs was 13.3 $ng.m^{-3}$. The ratio BeP/(BeP+BaP) in all aerosol samples from Celakovice was less than 0.5 indicating emission of aerosols from local sources. A carbon preference index (CPI) was in the range 0.98 – 1.09, which indicates vehicular emissions and other human activities as emission source of aerosols collected in Celakovice.

Analysed saccharides prove burning of biomass as emission source but they also indicate other emission sources of aerosols such as resuspension of soil as well different biological sources (spores, pollens, fungi, microorganism metabolites, fragments of plants).

Strong correlation was found between PM1, MAs, alkanes, hopanes, steranes and PAHs which proves combustion of organic material and traffic as main emission sources. Correlation between retene and dehydroabietic acid (combustion of soft wood); picene, hopanes and PAHs (combustion of coal) was also strong. However, correlation between pristane/phytane and steranes (traffic) was weak.

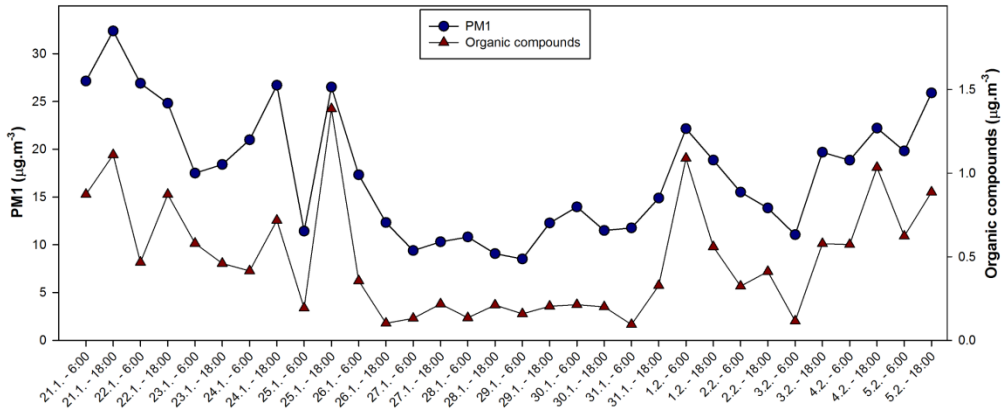

Fig. 2. Mass concentrations of PM1 (µg.m^{-3}) and sum of analysed organic compounds (µg.m^{-3}) in Celakovice.

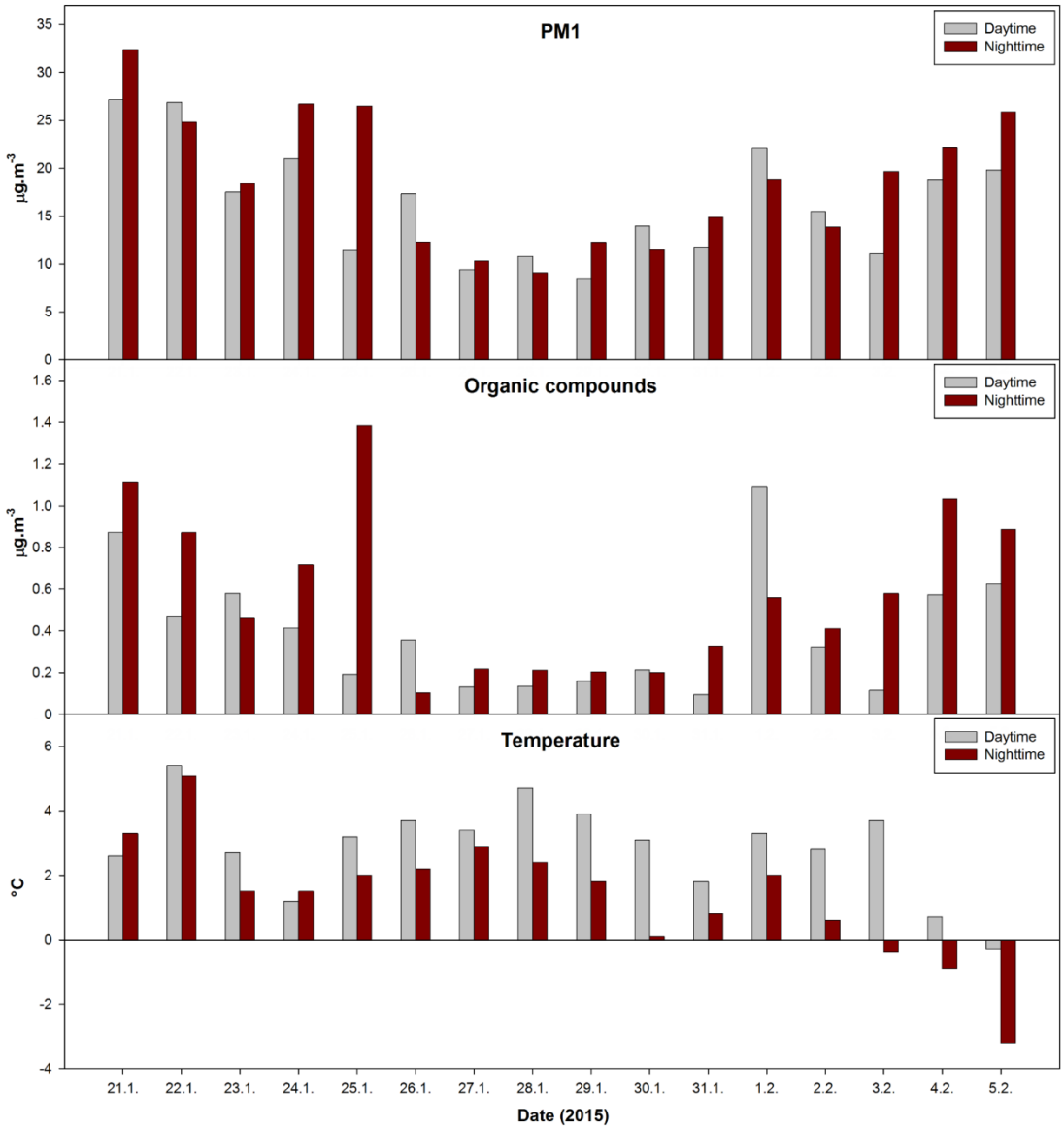

Fig. 3. Mass concentrations of daytime/nighttime PM1 (µg.m^{-3}), sum of analysed organic compounds (µg.m^{-3}) and temperature (°C) in Celakovice during sampling.

4 Conclusions

Combustion of biomass (especially soft wood) and coal belong to the biggest emission sources of organic compounds bound to aerosols collected during winter campaign in Celakovice. The composition of PM1 aerosols sampled in Celakovice was affected both by local emission sources and by regional transport of polluted air from villages nearby Celakovice and at larger distances from Celakovice. Finally, long-range transport from neighbouring countries might also contribute to the composition of aerosols collected in Celakovice.

Acknowledgement

This work was supported by Grant Agency of the Czech Republic under grant P503/12/G147 and by Institute of Analytical Chemistry of the CAS, v. v. i. under an Institutional research plan No. RVO:68081715.

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P19 OPTIMIZATION AND COMPARISON OF VARIOUS CE/FA VARIANTS FOR STUDY OF DRUG-PROTEIN INTERACTIONS

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Summary

The interactions of ligands with proteins are involved in regulation many processes in all forms of life. Besides the endogenous ligands the exogenous compounds represent also important interacting partners. Probably the most important group of exogenous compounds constitute the drugs. The study of drug-protein interactions is essential both for drug discovery, and also for therapeutic purpose. It is therefore highly important to estimate the drug binding ability to various proteins, not only in the early stages of drug discovery, but also in clinical practice. In this contribution the strength of interactions between model drugs – tolbutamide, lidocaine and diclofenac, and human serum albumin were measured by capillary electrophoresis-frontal analysis. Two detection approaches – UV and MS were utilized and obtained binding constants were compared. Application of MS detection resulted in increasing of detection sensitivity that is particularly beneficial for estimation of affinity constants of poorly soluble compounds.

1 Introduction

The interactions between drugs and human blood proteins, primarily human serum albumin (HSA) represent very important example of interaction pairs in living processes because they have a significant impact on free drug concentration in blood, which is responsible for therapeutic effect. As a result the study of interactions between drugs and HSA represents an essential part of the drug pharmacokinetics and pharmacodynamics.

One of promising techniques suitable for the study of such interactions is capillary electrophoresis (CE). Various approaches based on CE have been developed for these purposes [1]. Based on our previous experiences [2], the capillary electrophoresis-frontal analysis (CE/FA) was selected in this study since it brings many advantages like low sample consumption, fast analyses and possibility of using near-physiological conditions for studied interactions. The CE/FA approach typically employs UV detection. However the utilization of MS detection brings in most cases increasing of detection sensitivity, which can be especially useful for evaluation of binding constants (K_b) of poorly soluble compounds. In this consequence the possibility of CE/FA and MS combination for K_b determination between model drugs – tolbutamide (TB), lidocaine (LD), and diclofenac (DC) and HSA were investigated.

2 Experimental

Analyses were performed using Agilent 7100 CE system (Waldbronn, Germany) with the bare fused silica capillary with I.D. 75 μm, length 47 cm (PolymicroTechnologies, Phoenix, AZ, USA) termostated at 25°C. The injection was 25 mbar for 30 s (139 nL, 7 % of capillary volume). Analytes were detected at 200 nm under voltage of 5 kV applied in normal polarity. Ammonium acetate 75 mM, pH 8.5 was used as a background electrolyte (BGE).

MS detection was performed with quadrupole – time of flight Bruker maXis impact QTOF MS (Bruker Daltonics, Bremen, Germany) instrument in the positive ionization mode. The CE and MS was connected via the sheath liquid co-axial ESI interface and creating of electrospray was supported by solution consisted of MeOH-water (1:1) which was delivered at a flow rate of 4 $\mu L \cdot min^{-1}$. The nebulising pressure of nitrogen was set at 0.3 bar, the drying gas flow rate at 5 $L \cdot min^{-1}$, the drying gas temperature at 180 °C and the electrospray voltage was set at 4.5 kV. Analytes were detected as protonated molecular ions $[M+H]^+$ with monoisotopic mass 271.111, 235.180 and 296.024 for TB, LD and DC, respectively. CE/FA-MS analyses was evaluated from extracted ion electropherograms with width accuracy ± 0.005 m/z. Quantification was based on external calibration curve – standards of drug without protein injected into the capillary and analysed.

3 Results and Discussion

As mentioned above, the main objective of this study was to test the possibility of CE/FA and MS combination for determination of K_b between model drugs and HSA, and compare their values with those obtained by more convenient CE/FA-UV. In this

consequence it was predominantly necessary to find an appropriate BGE compatible MS detection.

Primary experiments were performed on the TB-HSA pair. Ammonium acetate was chosen with this regard, because this BGE is MS-friendly and its parameters are close to physiological condition. Its simple one factor optimization was performed to fulfil the basic demand of the CE/FA-UV i.e. the resolution between free drug and HSA + drug-HSA complex. Typical electropherograms are shown in Fig. 1. Estimated log K_b 4.28±0.18 was in perfect agreement with previously obtained values using phosphate (log K_b 4.12±0.11) and borate (log K_b 4.05±0.02) BGEs [3,4].

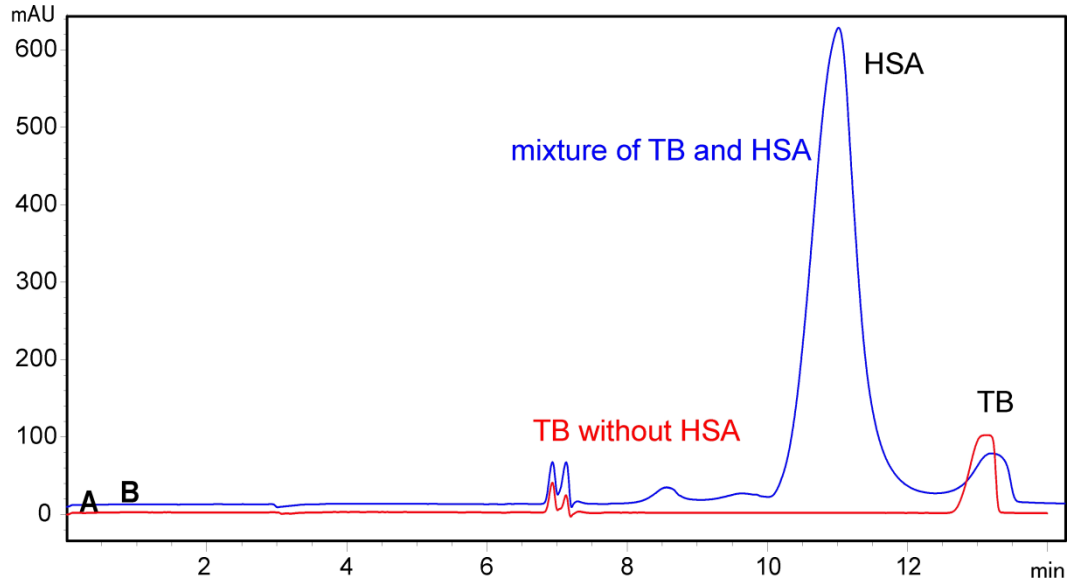

Fig. 1. Typical electropherograms of CE/FA-UV with TB. Concentration of TB was 400 µM and concentration of HSA was 75 µM. A) Analysis of TB standard. B) Analysis of TB standard mixed with HSA.

Identical acetate BGE was then used for CE/FA-MS measurements (Fig. 2). Established value was slightly differing – log K_b 5.06±0.04 from the value from obtained by CE/FA-UV log K_b 4.28±0.18.

Similarly the K_b for LD and DC were measured in acetate BGE by CE/FA-MS. The estimated K_b values (LD log K_b 3.14±0.08 and DC log K_b 5.22±0.10) were in the same order as data from UV approach using totally different BGE LD log K_b 3.29±0.07 and DC log K_b 4.41±0.01 – 150 mM sodium borate + 200 mM boric acid, pH 8.5 [4].

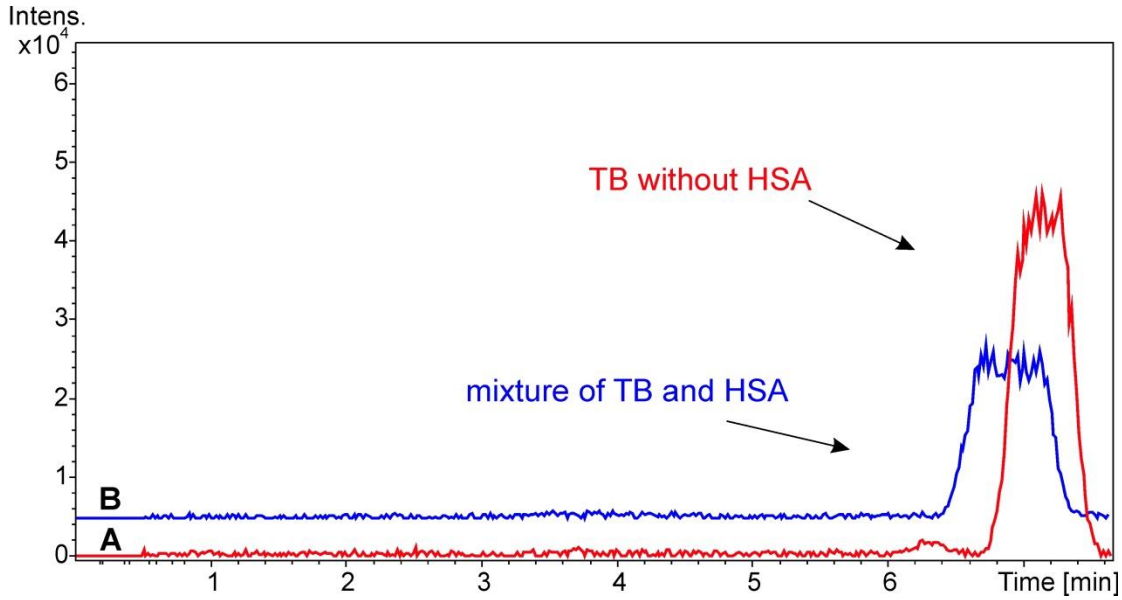

Fig. 2 Typical extracted ion electropherograms of CE/FA-MS with TB. Concentration of TB was 800 nM and concentration of HSA was 10 µM. A) Analysis of TB standard. B) Analysis of TB standard mixed with HSA.

4 Conclusions

The K_b is commonly used to describe the strength of binding between drug and protein that has a significant effect on the pharmacological activity of the drug. Many CE based approaches including CE/FA can be used for this purpose. Even though CE is well known for its many advantages in this regard, the sensitivity of CE analyses is sometimes insufficient for many drug-protein pairs. It is especially true in the case of poorly soluble drugs where low concentrations have been used for such studies. So the possibility of using CE/FA in combination MS detection was tested. What is more in contrast with classically used UV detection only drug can be detected so optimization for other drugs in future applications can be trivial.

Acknowledgement

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P20 SIMPLE ROUTE OF CASPASE-3 FRET SENSOR SYNTHESIS USING "CLICK CHEMISTRY"

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Summary
Programmed cell death or apoptosis is regulated process of cell suicide. The central role in apoptosis play cysteine proteases called caspases. Caspases recognize tetra-peptide sequences Asp-Glu-Val-Asp (DEVD) on their substrates and hydrolyze peptide bonds after aspartic acid residues. Various techniques for the determination of caspase-3 are commercially available e.g. Enzyme Linked Immuno-Sorbent Assay (ELISA), Western blotting or flow cytometric analysis. The products of the cleavage can be detected by spectrophotometry, fluorimetry, chemiluminescence (CL) or ELISA. In this work, we suggested fluorescent sensor based on easily prepared Förster Resonance Energy Transfer (FRET). We use very simple chemistry called "click". This type of chemistry takes advantages of quickness, simplicity and cheapness. "Click chemistry" is based on usage of various functional group and cross-linkers to combine individual molecules together.

1 Introduction

The morphological features of apoptosis include changes in plasma membrane asymmetry and attachment, condensation of cytoplasm, nucleus and internucleosomal cleavage of DNA. In the final stage cell gets converted into "apoptotic bodies" which is rapidly eliminated by phagocytosis without eliciting inflammation in the surrounding areas. The core component of the apoptotic machinery is a proteolytic system involving a family of proteases known as caspases. Caspases are essential in cells for apoptosis, one of the main types of programmed cell death, in development and most stages of adult life. Failure of apoptosis results in the tumor development and autoimmune disorders. However uncontrolled apoptosis occur in ischemia and Alzheimer´s disease. This has boomed interest in caspases as potential therapeutic targets. The caspases are cysteine proteases which cleave the peptide bond C-terminal to aspartic acid residues [1-5]. There is a lot of analytical method for detecting caspase-3 activity like Western blotting, flow cytometric analysis or ELISA with colorimetric/fluorimetric detection [6]. Plenty of techniques were recently developed for detection of caspase-3 in cells with high spatio-temporal resolution e.g. fluorescence resonance energy transfer (FRET) based-assay [7, 8]. Förster (fluorescence) resonance energy transfer (FRET) is a widely prevalent photophysical process that occurs between a donor (D) molecule in the excited state and an acceptor (A) molecule in ground states. FRET is one of the few experimental techniques that are able to detect and define distance between molecules, molecular dimensions, proximities

change with time, heterogeneous molecular conformations etc. [9]. We use system based on quenching of fluorescence. Preparation of sensor is based on "click chemistry". This chemistry exploits various types of cross-linkers which are small chemical compound with reactive groups to combine different molecules together.

2 Experimental

2.1 Chemicals

QDs were purchased from Life Technologies (Qdot® 565 ITK™ Amino (PEG) Quantum Dots) and quencher BHQ-2 OSu modified was purchased from Biosearch Technologies. Peptide sequence H_2N-SGDEVDGK-COOH was purchased from Clonestar Peptide Service. Conjugation reaction were done in sodium carbonate buffer (pH = 10.5; c = 50 mM). Na_2CO_3 (p.a.) was purchased from Lach-Ner. Cross-linkers like carbodiimide (EDC), succinimide (sulfo-NHS) and maleiimide (sulfo-SMCC) were purchased from Sigma Aldrich. 18.2MΩ × cm ultrapure water was produced by Neptune Purite Ultimate. As desalting columns were used SPE columns (SDB-L, 3 ml/200 mg) from Phenomenex®.

2.2 "Click chemistry"

At first, peptide sequence (1 mg/ml) was dissolve in 50 mM carbonate buffer at pH = 10.5. 20 µl of this peptide was mixed with 31.9 µl succinimide modified quencher BHQ-2 OSu (0.19 mg/380 µl in DMSO) and allowed reacting for 1 hour at room temperature. For conjugation reaction between aminated QDs and quencher modified peptide sequence were used zero-length cross-linkers like EDC/sulfo-NHS. Results were checked by CZE-LIF, MS and fluorescence spectra measurement.

3 Results and Discussion

We have designed sensor for caspase-3 determination in individual apoptotic cells. This sensor is based on FRET, were oscillating electrons, in donor in our case quantum dot, exchange energy with acceptor dipole (BHQ-2 OSu modified quencher) with similar resonance energy via the chain peptide sequence. Our modified BHQ-2 quencher absorbs transported energy and is able to quenched fluorescence. But after cleavage of the bond between quencher and DEVD sequence in presence of Caspase-3 in real samples, there is no non-radiative FRET transfer, quencher doesn´t absorb energy and QD emits light at given wavelength (Fig. 1).We used aminated QDs with maximum emission wavelength 565 nm.

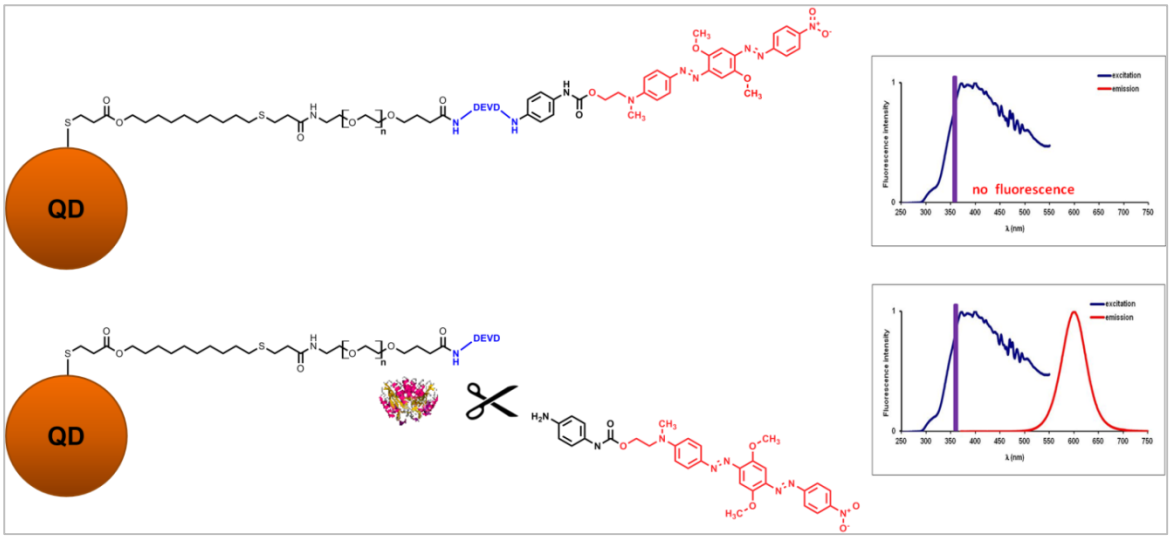

Fig. 1. Model of caspase-3 sensor with DEVD sequence; A) Model of caspase-3 sensor, without cleavage of DEVD chain and FRET transfer with quenching of fluorescence. B) Cleavage of bond between DEVD and quencher and no FRET transfer with QD´s emission of light.

Conjugation reaction between DEVD sequence and succinimide modified BHQ-2 quencher is one of the examples of "click" chemistry using zero-length cross-linker; in this case succinimide cross-linker is bonded to BHQ-2 quencher. The resulting conjugate was successfully desalted using SPE columns. The peak of conjugate is demonstrated in MS spectrum (Fig. 2).

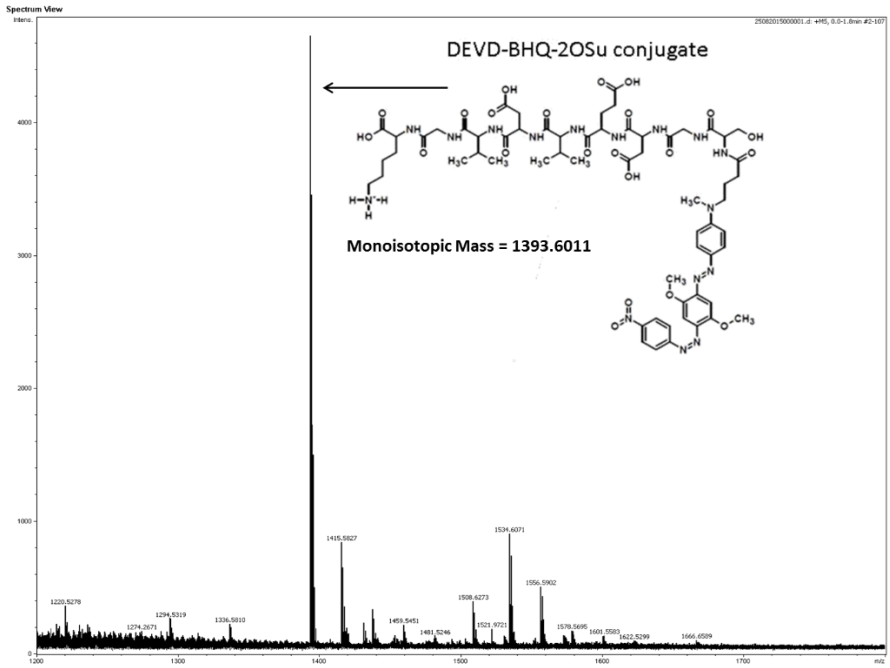

Fig. 2. ESI-TOF-MS analysis. m/z: 1393.6011 = product of reaction between DEVD sequence and BHQ-2OSu (50% ACN, 1% HCOOH).

4 Conclusions

We designed new sensor based on FRET to determined caspase-3 in individual apoptotic cells. Preparation of this sensor is based on "click chemistry" using various zero-length cross-linkers, which in this case is very fast and simple method to achieve required product. Using of this sensor offer us possibility for very fast analysis of samples without sample destroying by lysis. Results can be analyzed by fluorescence microscopy or spectrofluorometric measurements.

Acknowledgements

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P21 LC-MS ANALYSIS OF CHOSEN PHOSPHATIDYLCHOLINES IN HUMAN PLASMA

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Summary
Phosphatidylcholines (PCs) were studied as potential biomarkers of Alzheimer's disease by high performance liquid chromatography coupled to mass spectrometry (HPLC-MS). PCs were extracted from human plasma, separated by HPLC using several chromatographic columns, mobile phases and gradients. Detection was performed by various MS techniques while the final analyses were provided by quadrupole ion trap. Additionally, phosphatidylcholines were quantified by the standard addition technique and the extraction recoveries and matrix effect of plasma were calculated.

1 Introduction

Alzheimer's disease (AD) is a serious neurodegenerative disease, characteristic by memory losses and inability to carry out everyday activities [1]. The exact cause of the disease is still unknown and the treatment in later stages is mostly unsuccessful. Therefore, there is a demand for reliable molecular biomarkers to predict the disease or at least reveal it in early stages. As the affected organ, brain, is mostly formed by lipids, the search for biomarkers is focused on different lipid classes nowadays. Typical lipidomic analysis consists of several steps – extraction of lipids from the biological sample, their separation, detection and finally identification and quantification typically by MS [2]. Though MS is an efficient tool for molecule analysis, in lipidomics it is limited by the existence of high number of different lipid isobars and isomers, characterized by the same molecular mass. Consequently, for reliable lipid identification and quantification, other techniques have to be introduced, such as HPLC or nuclear magnetic resonance [3]. PCs are the main components of biological membranes and they have already shown their potential as biomarkers of AD [4, 5].

2 Experimental

2.1 Biological material, standards and chemicals

Human plasma was obtained from the University Hospital in Olomouc. Five PC standards were purchased - PC (16:0/16:0), PC (18:0/18:1), PC (17:0/17:0), PC (16:0/18:2) and PC (18:0/18:2) (from Avanti Polar Lipids, USA). Other used chemicals included 98-100%

formic acid, methanol (MeOH), ethanol (EtOH), acetonitrile (ACN), ammonium acetate, isopropyl alcohol, ammonium fluoride and LC-MS water. All chemicals were p.a. grade.

2.3 LC-MS instrumentation

PCs were analysed by reversed phase HPLC Dionex Ultimate 3000 RSLC (Thermo Scientific, USA) with the column Cortecs C18 (Waters, Ireland) coupled to MS QTRAP 5500 (AB Sciex, USA). MS/MS experiments were performed by 6540 UHD Accurate Mass QTOF.

2.4 LC-MS Settings

During the method development, different mobile phases (MP), gradients and settings were tested. Concerning final chromatography settings, the column temperature was 50 °C, MP flow rate 0.5 mL/min and sample injection volume 1 μL. MP A was 0.1% formic acid in water with 5 % of MeOH, MP B was MeOH with 0.1% formic acid and 5 % of water. The total analysis time was 14 minutes, with the gradient starting on 60% of MP B, achieving 100% B in 1.5 min, followed by the isocratic elution for 9 min with the return of gradient to 60% B in 0.1 min and staying unchanged until the analysis end. PCs were measured by various MS instruments both in positive and negative ionization modes, MS and MS/MS. Q-Trap with electrospray ionization in negative multiple reaction monitoring mode (ESI- MRM) was used for the final analyses. Data was analysed by Analyst software (Sciex, USA).

2.5 Sample preparation

Plasmatic PCs were extracted by mixing of plasma with extraction solvent (ACN or MeOH-EtOH 50:50 v/v) followed by 5 min sonication, 15 min freezing, 5 min centrifugation (14 000 rpm) and supernatant collection. Extraction recoveries were calculated by the addition of standard PC (17:0/17:0) to the plasma before or after extraction (this PC is not naturally present in human plasma). Matrix effect was calculated from the experiment, where plasma was substituted with water. PCs in plasma were quantified by the standard addition technique. The 5000x diluted extracted plasma was spiked 1:1 (v/v) by PC standards to final standard concentrations 0-2000 nM. From acquired peak areas, calibration curve was constructed and the initial PC concentration in plasma was calculated.

3 Results and Discussion

Five PCs (chosen based on [5]) were analysed by LC-MS approach. Selected PCs were separated following the elution rule that PCs with shorter fatty acyl chains and more double bonds are eluted earlier than those with longer chains and less double bonds (see Fig. 1) [6]. Additionally, despite the same concentration of all PCs in the mixture, different ion intensities were observed, since PCs with shorter fatty acyl chains and more double bonds provide higher ionization efficiency than those with longer and less saturated chains [7].

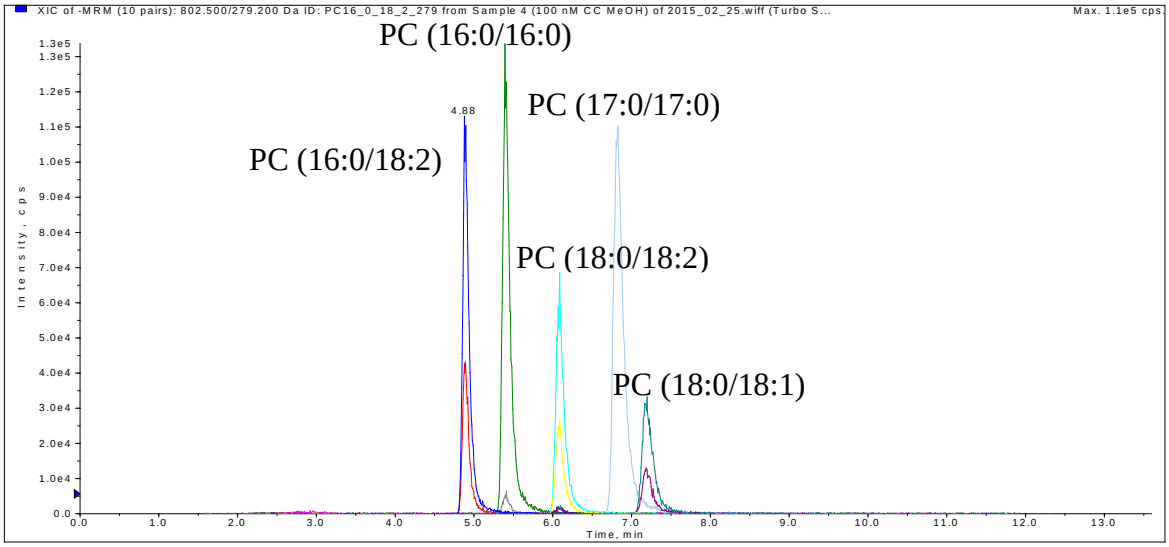

Fig. 1. ESI- MRM chromatogram of 100 nM PC standards in MeOH acquired by Q-Trap.

Concerning MS analysis, PCs were detected as hydrogen adducts in ESI+. In ESI- mode, they were detected as formic acid adducts which was caused by the formic acid in the mobile phase. Fragmentation (MS/MS) analysis showed the presence of 184 Da fragment in ESI+ belonging to the phosphocholine head which is similar for all PCs. In negative MS/MS, the first observed fragment was demethylated PC produced by the elimination of acetic acid from the initial adduct and other two fragments included fatty acyls, characterizing the individual PCs (Fig. 2).

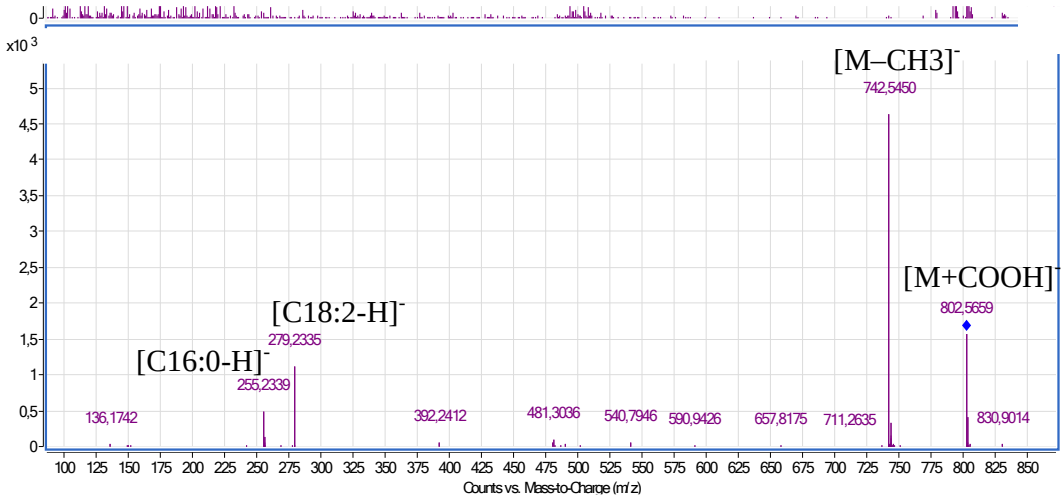

Fig. 2. ESI- MS/MS spectrum of PC (16:0/18:2) acquired by QTOF.

Regarding the extraction of PCs from plasma, MeOH-EtOH (50:50 v/v) was more efficient extraction solvent than ACN (extraction recovery 95 % ± 6 % vs 28 % ± 11 %.) which was related to the greater solubility of PCs in EtOH compared to ACN. Matrix effect of plasma was almost negligible since PCs act as ion suppressors rather than being

suppressed. The results of quantification of four selected PCs in human plasma are in Table 1.

Table 1. Quantification of PCs in plasma

PC	ESI- MRM product ion	plasma concentration (μM)
PC (16:0/16:0)	255.1	41.3
	480.3	60.6
PC (18:0/18:1)	281.3	6.9
	283.2	13.5
PC (18:0/18:2)	279.3	172.2
	283.3	15.3
PC (16:0/18:2)	279.2	300.1
	255.2	218.7

4 Conclusions

In this work, a suitable LC-MS method for analysis of phosphatidylcholines was developed. During method optimization, two extraction solvents, several chromatographic columns, mobile phases and gradients were tested and various mass spectrometer instruments were introduced. The developed method for Q-Trap mass spectrometer was able to separate and detect chosen PCs reliably and quantify them in human plasma.

Acknowledgment

The research was supported by the University of Eastern Finland and by the Institute of Molecular and Translation Medicine in Olomouc (NPU I (LO1304)).

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P22 METHOD DEVELOPMENT FOR BASELINE SEPARATION OF CYANOBENZ[F]ISOINDOLES OF PROTEINOGENIC AMINO ACIDS BY CE-LIF

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Summary
Baseline separation almost all of proteinogenic amino acids which underwent reaction with naphthalene-2,3-dicarboxaldehyde and cyanide anion to form N-substituted 1-cyanobenz[f]isoindoles was achieved using BGE comprised of 35 mM $Na_2B_4O_7$, 55 mM SDS, 2.7 M urea, 1 mM BIS-TRIS propane and 23 mM NaOH, pH 9.8. The derivatization reaction was performed in-capillary by sequential injection of the sample and the reagents, mixed by diffusion and the resulting products were analyzed by CE-LIF.

1 Introduction

Monitoring of amino acids (AAs) is important to understand biological processes. However, one of the most sensitive analyses employing laser-induced fluorescence (LIF) detection is hindered by the fact that the most AAs do not possess intrinsic fluorescence. Derivatization of the AAs is thus required by either reactive fluorophores (e.g. FITC or NBD-X) or fluorogennic compounds, such as OPA, CBQCA etc., which form fluorophores during reaction with analytes [1]. Naphtalene-2,3-dicarboxaldehyde (NDA) is analog to OPA synthetized in order to overcome unfavorable characteristic of OPA derivatives like limited stability of the derivatives and excitation by UV light requiring expensive lasers. NDA reacts with primary amino group in the presence of cyanide anion acting as nucleophile forming N-substituted 1-cyanobenz[f]isoindole (CBI) which is both fluorescent and electroactive (Fig. 1). The CBIs have excitation maxima at approx. 250 nm, 420 nm and 440 nm, and emission at 490 nm with excellent quantum yields (Φ 0.5-0.8) [2].

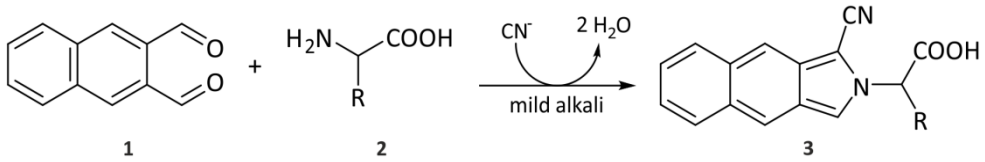

Fig. 1. Reaction scheme of the reaction of NDA (1) with α-AA (2) in the presence cyanide anion under mild alkali conditions yielding corresponding CBI (3).

The main goal of this work is to present baseline separation almost all of proteinogenic AAs by means of capillary electrophoresis (CE) with LIF detection, except for Pro and Cys which do not react with NDA or form nonfluorescent product [3]. According to the literature, some AA-CBI cannot be resolved under various separation conditions [4–6].

2 Experimental

2.1 Instrumentation

Agilent G7100 CE System (Waldbronn, Germany) coupled with Zetalif LED 480 LIF detector (Picometrics Technologies, Labège, France) was used in all experiments. The separation was carried out on a bare fused silica capillary of 50 μm ID (365 μm OD) with total length 71 cm and length from the inlet side of the capillary to the point of the LIF detection 50 cm. The new capillary was flushed with water, 1 M NaOH, 0.1 M NaOH and water for 5, 20, 10 and 10 min, respectively, to activate the surface of the inner capillary wall. Between runs, the capillary was flushed with methanol, 1 M NaOH and BGE for 3 min each with water flush (1 min) between the each flushing solution. The separation was conducted under the constant temperature 24°C and voltage 30 kV. The BGE was comprised of 35 mM $Na_2B_4O_7$, 55 mM SDS, 2.7 mM urea, 1 mM BIS-TRIS propane and 23 mM NaOH (pH≈9.8).

2.2 In-capillary derivatization

In order to automate the derivatization process, the inlet part of the capillary served as a reaction chamber. The each component of the reaction was injected sequentially to minimize the formation of side-products. At first, 1.5 mM NDA in 50% methanol was injected by pressure 50 mbar for 3 s, then the plugs of the same volume of AAs sample and 10 mM NaCN were injected. Each injected zone contained 50 mM $Na_2B_4O_7$/NaOH (pH 9.6) ensuring proper pH for the rapid reaction. The reagents were left to mix by diffusion and react for 5 min before the separation voltage was applied.

3 Results and Discussion

Since AA-CBIs cannot be effectively separated in a simple BGE, the micellar environment was induced by the addition of SDS into $Na_2B_4O_7$ buffer. Hydrophobic nature of CBIs caused formation of distribution equilibriums between the free solution and the micelles resulting in a broader separation window. Under wide range of the concentrations of SDS and $Na_2B_4O_7$ buffer, completely unresolved peaks of Tyr-CBI and taurine-CBI (common non-proteinogenous AA), and Phe-CBI and Val-CBI were

observed. As a result, additional additives were tested to improve the resolution. Organic solvents were promising, but they were eventually rejected as they considerably prolonged the separation time. The chaotropic agent urea efficiently resolved comigrating Phe-CBI and Val-CBI without affecting the separation time. The pair of peaks Tyr-CBI and taurine-CBI was resolved by the increase of the pH upon the addition of NaOH. Phenolic group of Tyr partly dissociates at the increased pH (pKa 10.1), thus the polarity of the Tyr-CBI increases and the distribution equilibrium between the free solution and the micelles was affected. Finally, cystine-CBI and Lys-CBI peak broadening was significantly reduced upon the addition of a small amount of BIS-TRIS propane into the BGE. Typical electropherogram of the model sample of AAs under optimal conditions is depicted on the Fig. 2. Resolutions among adjacent peaks is larger than 2.3 (Gln/Thr-CBIs; calculated with the peak widths at the half maximum) demonstrate baseline separation according to the criterion $R_s \geq 1.5$.

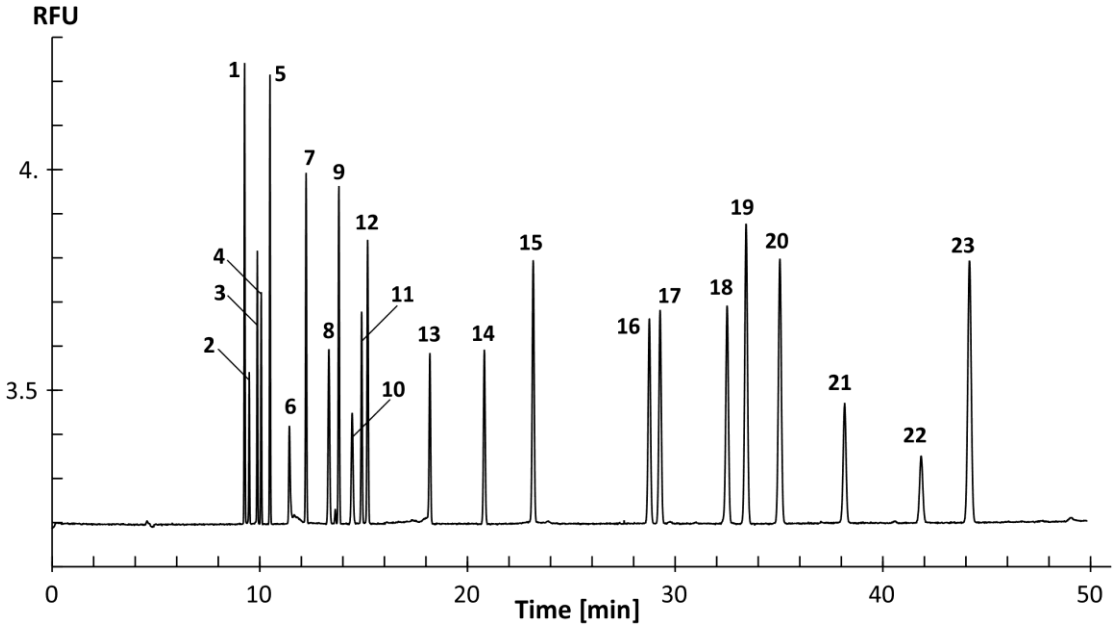

Fig. 2. Electropherogram of the model sample of AAs derivatized in-capillary and recorded under optimal separation conditions. Numbered peaks denoted to CBIs of Ser (1), Asn (2), Gln (3), Thr (4), His (5), cystine (6), Gly (7), Glu (8), Ala (9), Asp (10), Tyr (11), taurine (12), alanyl-glutamine (13), Val (14), Met (15), Ile (16), Trp (17), Phe (18), Leu (19), norleucine (20), Lys (21), Arg (23) and presumably cystine-bis-CBI (22). Concentrations of analytes were in range 5-30 μM.

4 Conclusions

AA-CBIs showed to be difficult to separate in a simple BGE. As a result, SDS and urea were added to enhance the separation efficiency. Besides peak broadening of cystine-CBI and Lys-CBI was minimized upon the addition of a small amount of BIS-TRIS propane. The baseline resolution (criterion $R_s \geq 1.5$) was achieved under presented conditions.

List of undefined abbreviation

BGE, Background electrolyte; CBQCA, 3-(4-Carboxybenzoyl)quinoline-2-carboxaldehyde; FITC, Fluorescein isothiocyanate; NBD-X, 4-halogen-7-nitrobenzofurazan; OPA, *o*-phtalaldehyde

Acknowledgement

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P23 CAPILLARY ISOTACHOPHORESIS WITH ESI-MS DETECTION: ULTRAHIGHLY SENSITIVE ANALYSIS OF DICLOFENAC AND IBUPROFEN IN WATERS

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Summary

This work shows that CE-MS can reach same sensitivity as HPLC-MS, when it is peformed in the ITP-MS mode. By combination of ITP-ESI-MS with an offline SPE preconcentration step and optimization of MS signal collection, LODs below $2x10^{-12}$ M can be reached, corresponding to of 0.6 ng/L of diclofenac (Dic) and 0.4 ng/L of ibuprofen (Ibu), compatible to that of SPE-HPLC-MS.

1 Introduction

The permanent ITP format ensures effective stacking analytes all the way until they reach the detector. Its combination with MS detection offers sensitive analysis and specific detection [1-4]. We have recently applied this technique to analysis of anionic

pharmaceuticals in waters [2] and show here what sensitivity can be reached with offline SPE and multiple ion monitoring.

2 Experimental

We used an Agilent 7100 CE system with a 100 μm id bare fused-silica capillary, CE-ESI-MS interface and 6130 single quadrupole mass spectrometer. For offline SPE, LiChrolut SPE 6 mL tubes (Merck) were used in an optimized procedure based on previous publications [5-7].

3 Results and Discussion

In the first part of the work, we have optimized fragmentor voltage and investigated monitoring of multiple ions. We found that this can bring an increase in sensitivity and contributes to specificity of the analysis (data not shown). The inclusion of an offline SPE step with a concentration factor of 50 and satisfactory recovery allowed to considerably increase sensitivity of the method and to apply it to water analysis. Fig. 1 show the analyses of drinking water spiked with $2x10^{-12}$ M Dic and Ibu (A), of drinking water (B), and of water from a small stream (C). The figure shows that the combined method allows to see traces of Dic in drinking water at a level around the LOD and to be at quantitation level for the analysis of surface water.

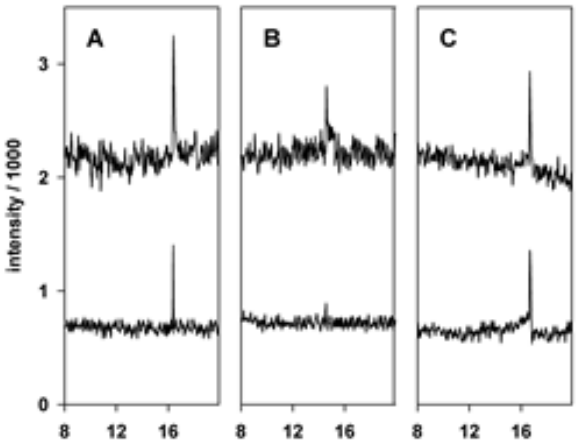

Fig. 1. Analysis of Dic (upper trace) and Ibu (lower trace). (A) drinking water spiked with 2.10^{-12} M Dic and Ibu, (B) drinking water, (C) water from a small stream. Injection 100 mbar/150 s, voltage of -25 kV, fragmentor 130 V, negative SIM data monitored for 7 m/z values for Dic and for 6 m/z for Ibu.

4 Conclusions

Optimized data collection and off-line SPE preconcentration further increase the sensitivity of ITP-ESI-MS by two orders of magnitude, with LOQ around $5x10^{-12}$ M which brings the SPE-ITP-MS method to the sensitivity level of SPE-HPLC-MS [7].

Acknowledgement
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P24 CHARACTERIZATION OF THE BINDING OF ANTIDIABETIC DRUGS TO HUMAN SERUM ALBUMIN BY MEANS OF CE-FA

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Summary
Antidiabetic drugs are prescribed as a treatment for *diabetes mellitus* to help lower glucose levels in the blood. With the exceptions of insulin, exenatide, liraglutide and pramlintide, all are administered orally and are thus also called oral hypoglycemic agents or oral antihyperglycemic agents. There are different classes of antidiabetic drugs, and their selection depends on the type of the diabetes, age and situation of the person, as well as other factors like development of blood sugar levels throughout the day, the presence of other diseases and unsuitable drug combinations. They are believed to primarily bind in blood to human serum albumin (HSA). This study used the capillary electrophoresis-fontal analysis (CE-FA) to examine the binding of antidiabetic drugs (tolbutamide, chlorpropamide, acetohexamide, carbutamide) to HSA.

The binding constant (K_b) was measured under physiological conditions. The log K_b values obtained by means of CE-FA have a good repeatability. Apparent K_b values obtained using nonlinear regression decrease in the sequence acetohexamide > tolbutamide > chlorpropamide > carbutamide.

The results provide a more quantitative picture of how these drugs bind with HSA and illustrate how CE-FA can be used to examine relatively complex protein-drug interactions.

1 Introduction

The antidiabetic drugs are divided into several classes. This study examined the class of sulfonylurea derivatives that belongs to secretagogues that increase insulin output from the pancreas. Sulfonylureas were the first widely used oral antihyperglycaemic medications and selected representatives (tolbutamide, chlorpropamide, acetohexamide, carbutamide) represent the first generation of these drugs [1].

The binding constant (K_b) is commonly used to describe the strength of binding between protein and ligand such as drug. The strength of the interaction has a significant effect on biological activity of the drug [2]. Various sulfonylureas (antidiabetic drugs) may induce hypoglycemia, therefore, it is important to understand in detail the pharmacokinetics and pharmacodynamics of these drugs [1]. The advantages of capillary electrophoresis for determination of K_b are very low sample consumption, high resolution and no requirements of the highly purified samples, immobilization or labelling of any of the interacting species. What is more the investigated interactions take place in a solution, which can be simulated physiological conditions [3, 4].

The main objectives of this study were to determine the binding parameters (binding constants and number of binding sites) of human serum albumin (HSA) with the selected drugs (tolbutamide, chlorpropamide, acetohexamide, carbutamide) using capillary electrophoresis-fontal analysis (CE-FA) under physiological conditions.

2 Experimental

2.1 Chemicals

All reagents were obtained in analytical grade. Sodium hydroxide, HSA, sodium phosphate, tolbutamide, chlorpropamide, acetohexamide and carbutamide were obtained from Sigma-Aldrich (Steinheim, German), hydrochloric acid was obtained from Fluka (Buchs, Switzerland).

The BGE in all experiments was the 67 mM phosphate buffer, pH 7.4. [2] All the 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).

2.2 Instrumentation

All experiments were performed in an Agilent ^{3D}Capillary Electrophoresis System (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array UV-Vis detection system. The analyses were carried out in a 75 μm I.D., 375 μm O.D. uncoated fused silica capillary with length 48.5/8.5 cm (L_{tot}/L_{eff}) from Polymicro Technologies (Phoenix, AZ, USA) thermostated at 37 °C. The samples were injected into the capillary with a pressure of 35 mbar at the cathode for 5 s. Operational voltage of 10 kV was applied in reversed polarity and the detection wavelength was set to 214 nm (tolbutamide), 200 nm (chlorpropamide) or 250 nm (acetohexamide and carbutamide).

3 Results and Discussion

3.1 Optimization and validation of method

Firstly, CE-FA method had to be optimized for physiological conditions. The rate and sufficient repeatability of the analysis were important requirement of this method. Therefore the following parameters have been optimized – the capillary length, separation voltage, injection time, flushing procedure between runs.

The repeatability of analysis (run-to-run / day-to-day), linearity in concentration range 20 – 800 μM, detection limit (LOD) and quantitation limit (LOQ) were evaluated for all drugs.

3.2 Determination of binding constants

Optimized method was used to determination of binding constants of model systems HSA-tolbutamide, HSA-chlorpropamide, HSA-acetohexamide and HSA-carbutamide. The set of representative binding curves obtained for HSA-tolbutamide with CE-FA method is shown in Fig. 1. CE-FA provides good repeatability, RSD of log K_b value is lower than 2.8 %.

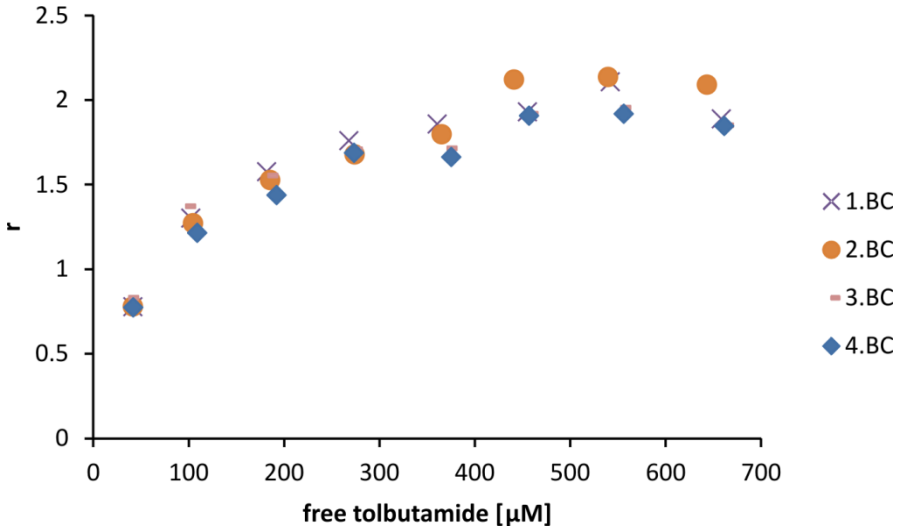

Fig. 1. Repeatability of binding curves (BC) obtained for HSA-tolbutamide system with the CE-FA method. Experimental conditions: BGE - phosphate buffer, pH 7.4, hydrodynamic injection (5 s, 35 mbar), separation voltage – 10 kV, λ = 214 nm, samples - mixture of 75 μM HSA with 100-800 μM tolbutamide.

4 Conclusions

The main aim of this work was to develop a method for K_b determination of sulfonylurea antidiabetics of first generation (tolbutamide / chlorpropamide / acetohexamide / carbutamide) and HSA. Apparent K_b values obtained using nonlinear regression were $1.34 \pm 0.36 \cdot 10^4$ L/mol for tolbutamide, $9.71 \pm 2.42 \cdot 10^3$ L/mol for chlorpropamide, $4.18 \pm 0.16 \cdot 10^4$ L/mol for acetohexamide and $9.53 \pm 1.07 \cdot 10^3$ L/mol for carbutamide.

Acknowledgement
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P25 CADMIUM TELLURIDE QUANTUM DOTS AS FLUORESCENT PROBE FOR DETERMINATION OF VALPROIC ACID

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Summary
In the present work, the fluorescence properties of CdTe quantum dots (QDs) were studied. The obtained results indicate that QDs exhibit the best emission of radiation (λ_{em} = 618 nm) after excitation at 250 nm within the pH range of 4.5 -7. A lower pH (1-4) caused a complete quenching of 0.05 mM CdTe QDs and also KCl (0.75 M) caused by a 94% decrease in the fluorescence signal of QDs. The addition of valproic acid (VPA) prevented this decline. Due to this finding, the influence of VPA and KCl on the fluorescence intensity of CdTe QDs was studied. VPA was found that could protect 0.05 mM QDs against quenching by 0.75 M KCl and that the fluorescence of 0.05 mM QDs treated with 0.75 M KCl is directly proportional to the concentration of VPA. This can

serve for indirect detection of VPA. The detection limit of VPA determination was 3 $\mu g.mL^{-1}$ and the calibration curve was linear within the range 10 – 950 $\mu g.mL^{-1}$.

1 Introduction

Valproate (VPA, valproic acid, $C_8H_{16}O_2$) belongs to the pharmacological class of antiepileptics of the 2nd generation [1]. VPA is a neuropsychiatric drug with a very broad clinical use. Broad therapeutic activity of VPA is mainly due to the action of neurotransmitters, transcription factors and gene expression [2]. Recently, it has become evident that VPA is also associated with anti-cancer activity [3]. The VPA and some VPA-analogues inhibit histonedeacetylases class I and IIa and modulate the biology of diverse tumour cell entities by inducing differentiation, inhibiting proliferation, increasing apoptosis by decreasing metastatic and angiogenetic potential [4]. The anti-tumour characteristics of VPA have been demonstrated [3]. Therefore, it is necessary to determinate the VPA in biological samples. There is a variety of techniques for the VPA detection, e.g.: ultra-performance liquid chromatographic system coupled with tandem mass spectrometry [5], high-performance liquid chromatography with electrochemical, fluorescence, diode array and/or tandem mass spectrometric detection [6-9], gas chromatography with flame ionization or electron-capture detection [10, 11], capillary electrophoresis with contactless conductivity or indirect UV detection [12, 13]. These techniques are precise but demanding sophisticated instrumentation and operator training. Therefore, we study a fast and simple method for detection of VPA based on changes in fluorescence intensity of CdTe quantum dots (QDs). It was demonstrated that VPA prevents quenching of CdTe QDs after addition of KCl. This finding can be used for sensitive and selective analysis of VPA.

2 Experimental

2.1 Preparation of quantum dots

QDs were prepared according to our previous work [14]. 2 mL of solution in small glass vessel were heated at 100 °C and 300 W for 10 min under microwave irradiation to prepare QDs of different colour. The concentration of the CdTe QDs stock solution was recalculated according to total concentrations of Cd(II) determined by atomic absorption spectrometry and diluted with distilled water.

2.2 Fluorescence measurement

Excitation wavelength was tested in the range of 220 – 570 nm. For all analyses, λ_{ex} = 250 nm and λ_{em} = 500 – 800 nm was used. The detector gain was set to 100. 100 µL of the samples were placed into a transparent 96 well microplate with flat bottom by Nunc. All measurements were performed at 25 °C controlled by the Tecan Infinite 200 PRO.

3 Results and Discussion

The ability of VPA to protect the CdTe QDs against quenching caused by 0.75 M solution of KCl (Fig. 1 A) was investigated. First, the fluorescence properties of 0.05 mM CdTe QDs were studied. CdTe QDs exhibit the best emission at 618 nm after excitation at 250

nm. CdTe QDs solution was titrated by 1 M HCl and 1 M NaOH and the fluorescence intensity was measured at different pH values. The QDs fluorescence intensity increases with increasing pH (4.5 –7), and slightly decreases by an additional increase in pH (7 - 12). The quenching of 0.05 mM CdTe QDs with 0 - 1.5 M KCl was studied. The QDs fluorescence intensity decreases with addition of 0.023 – 1.5 M KCl. 0.75 M KCl caused a decrease in fluorescence signal by 94%. Finally, the effect of VPA (0 – 15 mg.mL^{-1}), as a possible protective agent against quenching of QDs induced by 0.75M KCl, was studied.

50 µL of VPA (0 - 30 mg.mL^{-1}) was mixed with 25 µL of QDs (0.2 mM) and 25 µL of 3 M KCl. The prepared samples were immediately analysed. The measurement was repeated 4 times (in time 0, 3, 6 and 9 min). It has been shown that increasing concentration of VPA leads to the increase in fluorescence intensity of QDs (Fig. 1 B). It was possible to indirectly determine the VPA with a detection limit of 3 µg.mL^{-1} and the calibration curve was linear in the concentration range of 10 – 950 µg.mL^{-1} VPA (Fig. 1 C). The method was used for determination of VPA encapsulation in the liposome nanotransporter, applicable to increase the efficiency of treatment by this drug. It was found out that the determination was not significantly affected neither by the presence of the liposomal matrix nor by the presence of the 3% sodium dodecyl sulphate, which was used for liposome disassembling and VPA releasing.

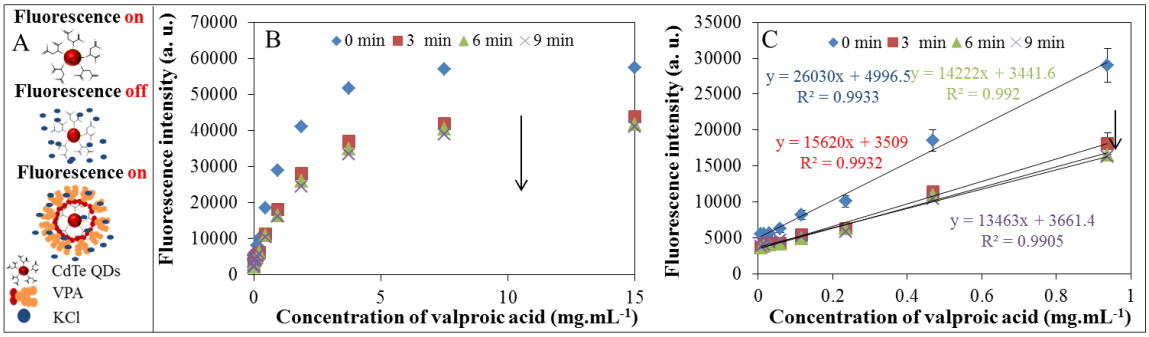

Fig. 1. A) Scheme of the interaction of CdTe QDs with KCl and VPA. B) Effect of 0-15 mg.mL^{-1} VPA on changes in the fluorescent signals of QDs in time 0, 3, 6 and 9 min. C) Calibration curves with a linear range of 10-950 µg.mL^{-1} VPA in time 0, 3, 6 and 9 min. (LOD = 3 µg.mL^{-1}, n =3).

4 Conclusions

In this work, we demonstrated that VPA could protect CdTe QDs against quenching induced by KCl. This ability can be used for indirect fluorescence detection of VPA. Further work will focus on the detection of VPA in various matrices (urine, blood plasma and liposomes).

Acknowledgement

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P26 MODERN METHODS FOR DETERMINATION OF BINDING CONSTANTS: CAPILLARY ELECTROPHORESIS VS. ISOTHERMAL TITRATION CALORIMETRY

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Summary

Strength of the binding between ligands (drugs) and proteins is commonly described by binding constant (K_b). These interactions have a significant effect on the biological activity, pharmacodynamics and pharmacokinetics properties of drugs. K_b can be determined by several methods. In this study capillary electrophoresis-frontal analysis (CE-FA) and isothermal titration calorimetry (ITC) are compared. K_b of systems

diclofenac-HSA and lidocaine-HSA were measured by both methodologies. The results of CE-FA and ITC are comparable and can be used for investigation of other binding parameters.

Advantages of CE-FA are very low sample consumption, short analysis time and it can be used in high-throughput screening of drugs. Using ITC we can obtain also thermodynamics parameters like enthalpy and entropy in single experiment, but disadvantages are large sample consumption and problem with estimation of weak affinity interactions.

1 Introduction

Binding of drugs to plasma proteins (particularly to the serum albumin) in the body is reversible process. Bound drug is not exposed to metabolism, but only free drug is considered to diffuse from the blood to the extravascular site and exhibits the pharmacological activity. Study of drug-protein interactions is thus important in clinical therapy and in the development of new drugs [1, 2].

Interaction strength between drug and protein is described by binding constant (K_b), which can be measured by several methods such as equilibrium dialysis, ultrafiltration, ultracentrifugation, spectrometry, affinity chromatography or electrophoresis and by calorimetric methods [3, 4]. In this study the applicability of capillary electrophoresis-frontal analysis (CE-FA) and isothermal titration calorimetry (ITC) for determination of K_b between drugs and human serum albumin (HSA) were investigated. HSA is the most abundant protein in the blood plasma, and it has two major drug binding sites located in subdomains IIA (Sudlow site 1) and IIIA (Sudlow site 2) [5, 6].

2 Experimental

2.1 Instruments

The CE-FA experiments were performed on Agilent ^{3D}Capillary Electrophoresis (Agilent, Waldbronn, SRN) with UV-VIS diode array detector. The fused silica capillary (Polymicro Technologies-Phoenix, USA) of 58.5 cm total length and 75 μm I.D. was used in experiments. The Microcal VP-ITC titrator (Malvern Instruments, Ltd, Worcestershire, UK) was used for the calorimetric titrations.

2.2 Chemicals

Investigated drugs (diclofenac and lidocaine) and HSA were obtained from Sigma-Aldrich (St. Luis, USA). Sodium hydroxide was obtained from Onex (Rožnov pod Radhoštěm, Czech Republic) Boric acid and sodium tetraborate and hydrochloric acid were purchased from Fluka (Buchs, Switzerland).

2.3 Solution and sample preparation

The buffer (BGE) was prepared by mixing 150 mM sodium tetraborate and 200 mM boric acid to resulting pH = 8.5. pH values were measured with an Orion Research EA940 (Orion Research-Waltham, USA).

Stock solutions of drugs (10 mM) were prepared in BGE and standard solutions were obtained from stock solutions by dilution with BGE to a desired concentration. Stock solution of protein (500 µM) was prepared daily in BGE.

2.4 Electrophoresis conditions

Before each analysis the capillary was flushed for 1 min with 1 M hydrochloric acid, for 1 min with water, for 2.5 min with 1 M sodium hydroxide, for 1 min with water and for 2 min with BGE. The capillary was thermostated at 25 °C, samples were injected at 35 mbar for 40 s and the voltage of 14 kV was applied for separation. Detection wavelengths were 276 nm for diclofenac and 214 nm for lidocaine.

2.5 Calorimetry conditions

HSA concentration in the ITC cell (1400 µL) was 75 µM. The drug concentrations used for injection were 4 mM (injection volume 8 µL, 35 times). Cell temperature was 25 °C and stirring speed was 307 rpm.

3 Results and Discussion

In CE-FA calibration curve was established from solutions contains variable amount of drugs in range 20-800 µM in absence of HSA. For determination of K_b the solutions with increasing drug concentration in range 50-800 µM and fixed total HSA concentration (75 µM) were measured. Concentration of free drug was measured from height of the plateau - H_f and calculated based on external drug standard in HSA absence - H_s. This is illustrated in Fig. 1.

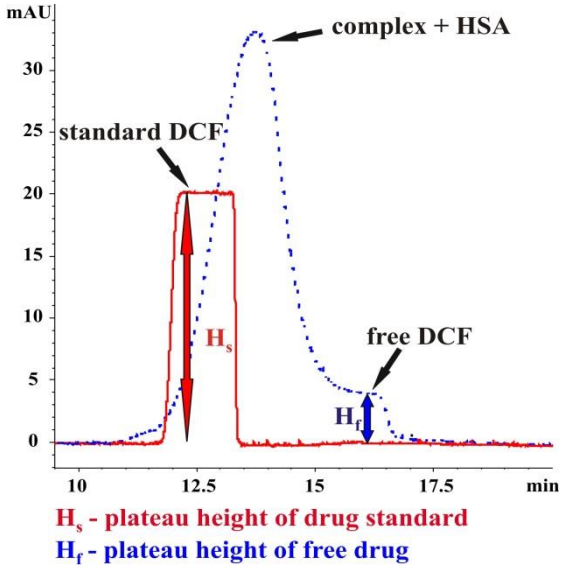

Fig. 1. Typical electropherograms of standard diclofenac (DCF) – solid line and mixture of DCF-HSA – dashed line obtained by CE-FA.

The bound drug concentration was calculated from difference between total and free drug concentrations. Binding parameters were determined by nonlinear regression

[7]. The obtained K_b values from CE-FA were 2.56 (± 0.07) · 10^4 L·mol^{-1} for diclofenac-HSA and 1.96 (± 0.32) · 10^3 L·mol^{-1} for lidocaine-HSA. In ITC absorbed or released heat is measured. The K_b value obtained from ITC were for diclofenac-HSA 1.22 (± 0.09) · 10^4 L·mol^{-1} and for lidocaine-HSA 1.28 (± 0.24) · 10^3 L·mol^{-1}. K_b values of each drug for both methods were measured in triplicate.

4 Conclusions

The K_b values of diclofenac or lidocaine binding to HSA were determined by CE-FA and ITC and were in good agreement. Both methods can be used for investigation of binding parameters without immobilization in solution. CE-FA has very low sample consumption and high resolution; disadvantage is adsorption of protein on capillary wall. Using ITC we can determine also thermodynamic parameters like enthalpy and entropy in single experiment, but there is the problem with large sample consumption and estimation of weak affinity interaction.

Acknowledgement

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P27 COMPARISON OF FOUR METHODS FOR DETERMINATION OF DOXORUBICIN

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Summary
Four different methods utilized for the determination of doxorubicin (HPLC-ED, DPV, spectrophotometry and fluorimetry) were compared using sixteen doxorubicin standards (5, 10, 20, 25, 35, 40, 50, 60, 75, 80, 100, 125, 200, 250, 500, 1000 μg.mL^{-1}) prepared in ACS water. The HPLC-ED method was found to be most suitable for determination of doxorubicin concentrations in its linear range of 5 – 1000 μg.mL^{-1}.

1 Introduction

Doxorubicin hydrochloride, is a cytotoxic anthracycline antibiotic isolated from cultures of fungus *Streptomyces peucetius* var. *caesius.* The most common use of doxorubicin in cancer therapy is for the treatment of various types of testicular cancer, leukemia, non-Hodgkin's and Hodgkin's lymphoma, osteosarcoma, neuroblastoma, and breast carcinoma [1]. Several methods have previously been reported for quantification of doxorubicin in biological fluids and tissues. Techniques such as voltammetry for urine [2, 3], high performance liquid chromatography (HPLC) for rat plasma and tissues [4], human plasma of cancer patients [5] were used. In this work a suitability of four different methods utilized for doxorubicin determination was compared.

2 Experimental

2.1 Preparation of doxorubicin standards

Doxorubicin standards were prepared by dilution of its stock solution (1 mg.mL^{-1} doxorubicin) with ACS purity water and calibration curves were measured. Sixteen different doxorubicin standards (5, 10, 20, 25, 35, 40, 50, 60, 75, 80, 100, 125, 200, 250, 500, 1000 μg.mL^{-1}) were used for comparison of four methods.

2.2 Determination of doxorubicin using HPLC-ED

Doxorubicin was analyzed by HPLC-ED (HPLC with electrochemical detection) according to [6, 7] with minor modifications. HPLC-ED system consisted of two chromatographic pumps Model 582 ESA (ESA Inc., Chelmsford, MA) (working range 0.001-9.999 $mL.min^{-1}$) and chromatographic column with reverse phase Kinetex 5µm EVO C18 (150 × 4.6 mm, Phenomenex, Inc. USA) and twelve-channel CoulArray electrochemical detector (Model 5600A, ESA, USA). Flow rate of mobile phase was 1 $mL.min^{-1}$. Mobile phase consisted of A: trifluoroacetic acid (80 mM) and B: 100% Met-OH. Detection was carried out at an applied potential of 900 mV.

2.3 Electrochemical detection of doxorubicin

Doxorubicin was analysed by differential pulse voltammetry (DPV) according to [8, 9] with minor modifications. All measurements were performed with an AUTOLAB Analyser (EcoChemie, Netherlands) connected to a 663 VA Stand instrument (Metrohm, Herisa, Switzerland). The three-electrode systems consisted of a glassy carbon electrode, an Ag/AgCl/3M KCl reference electrode and a platinum electrode serving as the auxiliary. Software GPES 4.9 was used for data analysis. Britton Robinson buffer pH 7 was used as supporting electrolyte. The parameters of the measurement were as follow: initial potential of 0 V, end potential of 1.8 V, step potential of 0.00495 V, modulation amplitude of 0.1 V, modulation time of 0.004 V, interval time of 0.1 V.

2.4 Absorbance measurement

The absorbance measurements were performed according to [10]. Briefly, 50 µl of various doxorubicin concentrations was pipetted into a UV-transparent microtiter plate well (Corning® Costar® 96-well plate, Corning, NY, USA). The absorbance spectrum (230-850 nm) of doxorubicin was measured using Tecan Infinite 200 PRO (Tecan, Männendorf, Switzerland). The absorbance maximum at 480 nm was used for determination of doxorubicin concentrations.

2.5 Fluorescence measurement

The fluorescence measurements were performed according to [11]. Briefly, 50 µl of various doxorubicin concentrations was pipetted into a UV-transparent microtiter plate well (Corning® Costar® 96-well plate, Corning, NY, USA). Emission spectrum (515-815 nm) of doxorubicin was measured using the excitation wavelength of 480 nm and Tecan Infinite 200 PRO (Tecan, Männendorf, Switzerland). The emission maximum at 600 nm was used for evaluation.

3 Results and Discussion

Using ten doxorubicin standards, calibration curves for four different methods (HPLC-ED, DPV, spectrophotometry and fluorimetry) were determined (Fig.1). The largest range for determination of doxorubicin concentration was observed using HPLC-ED (5-1000 $\mu g.mL^{-1}$), followed by the DPV method with a range of 10 to 1000 $\mu g.mL^{-1}$. The lowest range of doxorubicin concentration measurement was determined for both spectrophotometric and fluorimetric methods (5-250 $\mu g.mL^{-1}$). Regarding the LOD and

LOQ sensitivity of the methods, they were comparable with LOD ranging from 0.22 to 0.6 μg.mL^{-1} and LOQ from 5 to 10 μg.mL^{-1}. Coefficients of determination of calibration curves were also comparable, with R^2 in the range of 0.97 - 0.99. Percentage of successful measurements of 10 different standards with 5 repetitions of each standard within RSD=20% was also investigated. The best accuracy was observed using photometry (100%), followed by both fluorimetry and HPLC-ED (90%). The lowest accuracy (80%) was observed using DPV.

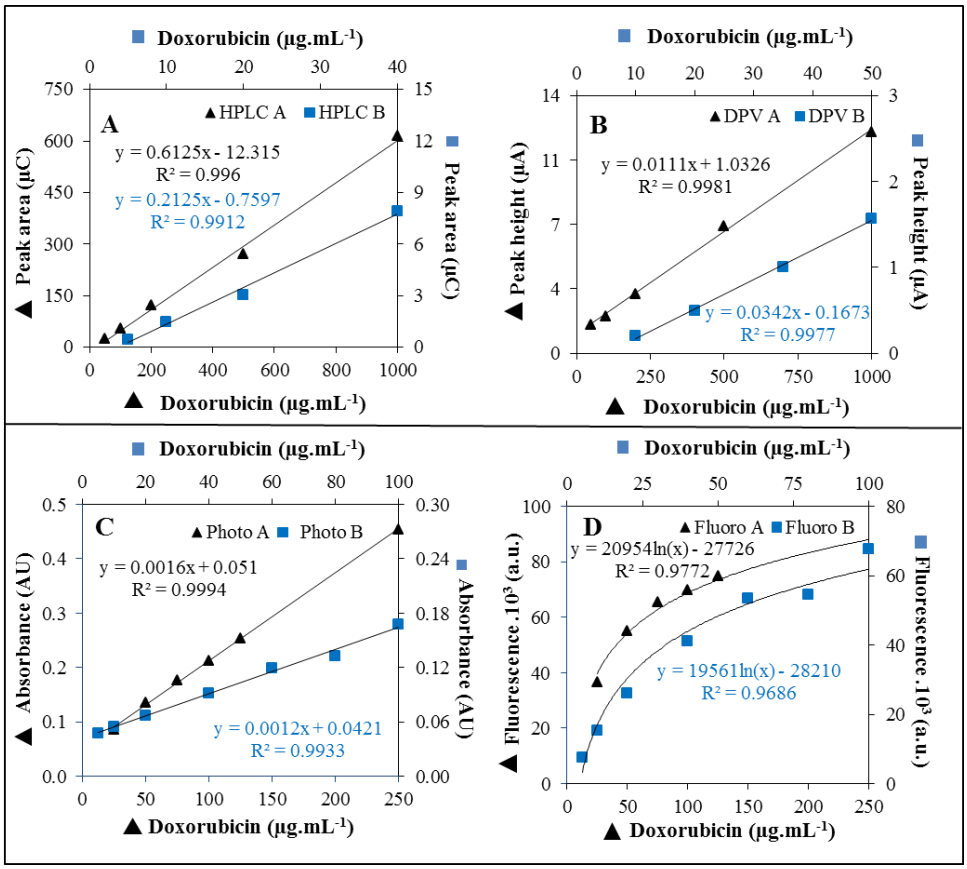

Fig. 1. Calibration curves of doxorubicin determination using A) HPLC-ED ▲ 50-1000 μg.mL^{-1}; ■ 5-40 μg.mL^{-1}, B) DPV ▲ 50-1000 μg.mL^{-1}; ■ 10-50 μg.mL^{-1}, C) spectrophotometry ▲ 25-250 μg.mL^{-1}; ■ 5-100 μg.mL^{-1}, and D) fluorimetry ▲ 25-250 μg.mL^{-1}; ■ 5-100 μg.mL^{-1}.

4 Conclusions

Of four methods tested for doxorubicin determination, the HPLC-ED method was found to be most appropriate, reaching the best analytical parameters. We suppose that this method could be, after further verification, suitable for use in clinical practice.

Acknowledgement

Financial support by GA CR (NANOCHEMO 14-18344S) is highly acknowledged.

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P28 DEVELOPMENT OF MICROCHIP PLATFORM FOR ELECTROPHORETIC SEPARATION OF OLIGOSACCHARIDE DERIVATIVES

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Summary
In this work, the microchip platform applicable in 2D system has been developed, characterized by separation of fluorescently labelled maltooligosaccharides and compared with the separations in capillary format.

1 Introduction

Oligosaccharides play a significant role in posttranslational modifications of proteins. The changes in protein glycosylation may serve as an indicator of some diseases [1]. For determination of glycans presented in organisms at low concentrations, fluorescent

labelling is usually required, which together with the application of microchip system may be used for their fast and efficient electrophoretic separation. In present work, development and characterization of such microchip platform is presented and compared to the capillary system with similar dimensions.

2 Experimental

Capillary separations were performed on Agilent ^{3D}CE electrophoretic instrument (Palo Alto, CA, USA) in volatile BGEs (formic and acetic acid, ammonium formate, ammonium acetate). Microchip was prepared by photolithography and wet etching procedure as described elsewhere [2] and mounted in plastic frame fabricated using 3D printing technology; connection to high voltage power supply (Spellman, Hauppauge, NY, USA) was utilized using Teflon reservoirs filled with background electrolytes. The channels have 25 and 50 μm x 60 μm profile with 65 mm length. Maltooligosaccharides (maltose to maltoheptaose) were labelled with 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and used for characterization of performance of developed system. For the detection, the slightly modified design of 3D printed LED-induced fluorescence detector was used [3].

3 Results and Discussion

The microchip platform intended for application in 2D system in combination with liquid chromatography was fabricated with inlet ports of etched channels on the edges of chip. Thus the electrodes and BGE reservoirs can be easily placed in plastic frame holding the glass device and sealed with o-rings or using Teflon inserts. We have prepared the plastic frame and we have compared Ohm's law dependencies for chip and capillaries. Results suggested that glass microchip can more easily dissipate Joule's heating and thus higher voltage can be used resulting in faster separation of target compounds. The efficiency of injection using "Z-shape" channel was assessed using 3,7-bis(dimethylamino)-phenolthiazine-5-ium chloride organic dye solution. Finally, the platform was combined with LED-induced fluorescence detector mounted on X,Y,Z-translational stages for precise alignment of excitation beam and the system was tested using ANTS labelled oligosaccharides yielding sub-minute range separation of all homologues.

4 Conclusions

In this work, the instrumentation for electrophoretic separation of fluorescently labelled oligosaccharides in microchip format has been developed. The microchip and plastic frame design allow application of LED-induced fluorescence detection and also potential coupling in 2D separation system with capillary liquid chromatography.

Acknowledgement

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P29 SPECTROSCOPICAL TECHNIQUE USING A RADIOFREQUENCY PLASMA PENCIL-TYPE DISCHARGE

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Summary
A tool called plasma pencil, using a barrier capacitively coupled radiofrequency discharge and operated in a continuous mode, has been originally constructed for the thin layer deposition and modification, but the other frequency, mode and use are also possible. Then it was demonstrated, that it can be also used as an alternative excitation source instead of inductively coupled plasma (ICP) as its partial replacement in some cases. Plasma pencil used in this study is created by a thin quartz tube with aerosol inlet, electrically powered by the radiofrequency generator and mechanically fixed by a ground and a power ring electrode. Operation mode was continuous created by a 13.56 MHz sine wave only or pulsed created by a carrying wave modulation by rectangular pulses with frequency 22 kHz and duty cycle of 90 %, which guaranteed good discharge stability. Plasma pencil device is smaller, constructionally simpler, and more versatile. The advantage is also lower purchase and operational cost. On the other hand it provides higher limits of detection and limited range of determinable elements in comparison with an ICP device. The energy transfer in the two electrode connection of the plasma pencil enhances its tolerance to an aerosol created by distilled water or by an aqueous analyte solution. Here the water aerosol was created by a concentric nebulizer connected to a Scott spray chamber. The plasma pencil was successfully used as an effective excitation source for the atomic emission spectrometry of some transition metals and especially of the alkali metals and alkali earth metals. Calibration dependences are linear in the range of three orders of magnitude. The pulsed regime study shows that the signal intensities are significantly lower in the pulsed regime than in the continuous regime except for calcium. It can be explained by the different axial lines profiles and by the calcium oxide and hydroxide formation in the shorter distance from the sample aerosol inlet to the discharge. The pulsed regime causes switching off and shortening of the discharge. It could be possibly explained by the widening or shortening of the rectangles during the

duty cycle changes. Elements lines have various excitation potentials and the measured emission intensities show various effects. Limits of detection and the sensitivity are different for a one-element and for a multi-element solution and are given by the mutual interactions between ions and atoms, which cause some remarkable effects. The extent of these effects cannot be strictly and simply based on the ionization energies of the studied elements. Besides, plasma pencil has a cylindrical symmetry and an extent dependence of the measured intensities on the observation spot along the discharge axis is observed. Element emission lines with higher excitation energies have relatively stronger intensities next to the ground electrode located between aerosol inlet and discharge tail and the other lines are more intensive near the power electrode located at the discharge start just in front of the aerosol inlet. The difference between the ionization energy of one element and the upper level of the measured transition of the other element in mixture strongly influences measured intensities. Evidential depression and to less extent an increase of some emission lines are then observed. The axial emission profile is not substantially changed. The observed ef-fects of the intensity changes are lower if there is a big energy gap between the upper level of the measured transition and the ionization energy of the interacting element.

Acknowledgement
This work was supported by the project "CEITEC – Central European Institute of Technology" (CZ.1.05/1.1.00/02.0068) and by the project (CZ.1.05/2.1.00/03.0086) funded by European Regional Development Fund and project LO1411 (NPU I) funded by Ministry of Education, Youth and Sports of Czech Republic.

P30 THIOL-ENE-BASED MONOLITHIC ENZYMATIC MICROREACTOR FOR GLYCOPROTEIN DEGLYCOSYLATION

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Summary
Enzymatic microreactor was fabricated from thiol-ene polymer. Glycoprotein samples were deglycosylated by enzyme PNGase F which was immobilized on the monolith.

1 Introduction

Thiol-group reacts with allyles in step-growth radical reaction induced by UV-light which results in formation of solvent resistant polymers known as thiol-enes. When mixed with epoxy resins these materials are used in industrial coatings and adhesives.

Uncured thiol-ene mixture can be dispersed in immiscible solvent and cured by UV-light. Solvent and a phase boundary act as sort of lost mould for thiol-ene polymer, which can form a porous monolith.

If thiolic and allylic function groups are not mixed at 1:1 ratio, the resulting polymer is called off-stoichiometric thiol-ene. This material has excess of either thiols or allyles, which can be utilized in immobilization of biomolecules – immobilization on thiols in thiol-excess polymer or UV-induced allyl-thiol immobilization in ene-excess polymer. Amino-groups could be introduced this way by cysteamine (aminoethanethiol) [1].

Enzymatic monolithic reactor for deglycosylation was covered with hydrolithic enzyme *PNGase F*. Samples of glycoprotein *RNase b* were introduced into the reactor and products were analyzed by mass spectrometry.

2 Experimental

Master mould structures were micromilled in PMMA. Template moulds were cast out of PDMS and used for thiol-ene curing.

Thiol-containing monomer (in this case PETMP) was mixed with ene-monomer (TAIC) at intended ratio. 2,2-dimethoxy-2-phenylacetophenone was added as photoinitiator if weaker sources of the UV light were used. UV-cured thiol-ene parts were bonded placed in conformal contact by heat and additional UV-curing.

Thiol-ene emulsion for monolith was prepared by mixing thoroughly 1 part of thiol-ene monomer mix with 4 parts of methanol. The emulsion was introduced into the microchannel using syringe or pipette. After the polymer was cured by collimated UV-light, the unreacted liquid was washed out of the channel.

Two methods were used for enzyme immobilization. In chips with thiol-excess monoliths a solution of PNGase F was simply introduced into the unmodified channel and incubated overnight at 4°C.

In allyl-excess monolith the principles of click chemistry were used. The channel was filled by 2-(tert-butoxycarbonylamino)ethanethiol and exposed to UV light to initiate bonding between its thiol and allyl on monolith. The amino-group was then deprotected by 4M HCl.

Deprotected amino-groups were coupled with PNGase F by diketo agent. A solution of L-ascorbic acid was used instead of more common but toxic glutaraldehyde [2].

The solution of 1 mg/ml RNase b (50 mM ammonium bicarbonate buffer, 5 mM TCEP-HCl) was denatured by heating for 10 minutes at 100°C. Denatured and reduced glycoproteins were slowly introduced into the reactor and 20 µl samples were collected by applying vacuum on the outlet of the channel through trapping device.

For comparison, 2 µl of 500 U/ml PNGase F were added to 20 µl of the denatured solution of RNase b and incubated for 2 hours at 37°C according to the standard procedure recommended for PNGase F by manufacturer.

Samples treated in reactor and by standard procedure, as well as control sample of enzymatically untreated denatured working solution, were analyzed by HPLC-ESI-QTOF system (C2 purification column and Waters ESI-MS Synapt G2 Q-TOF mass spectrometry analyzer).

3 Results

3.1 Monoliths

Monoliths were characterized by Scanning Electron Microscopy (SEM). Monolith formed a network of uniform interconnected beads approximately 1 µm in diameter. These beads fused with the wall of the channel providing strong anchoring even if higher pressure was applied.

3.2 Deglycosylation

Analysis of mass spectra showed distinctive simplification in samples deglycosylated by PNGase F compared to the control sample of untreated glycosylated RNase b. This is caused by the loss of glycoforms during the deglycosylation process.

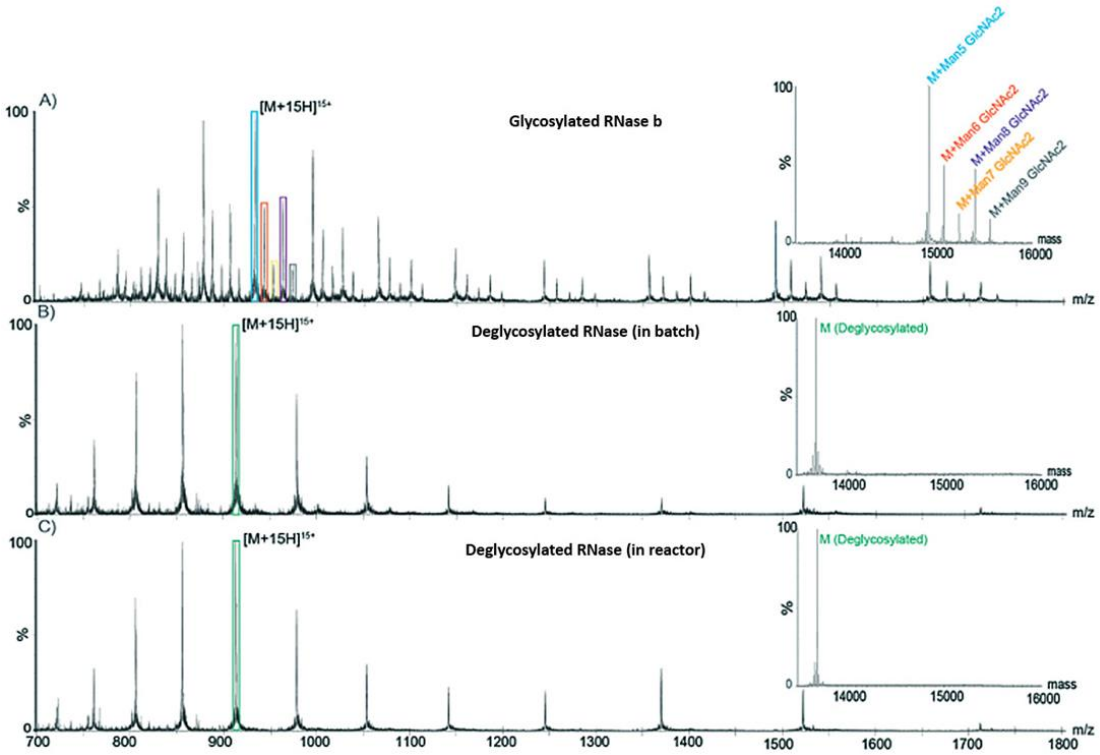

Fig. 1. Mass spectra - miniatures on the right side show deconvoluted spectra.
(MS by Nanna Bøgelund and Kasper D. Rand, University of Copenhagen, taken from [3] – slightly altered)

Mass spectra were deconvoluted to specify molar mass. Deconvoluted spectrum of glycoprotein showed 5 major peaks between 15 and 16 kDa. This corresponds with the

presence of 5 glycoforms. Spectra of RNase deglycosylated either on chip or in solution both showed single dominant peak at 13692 Da proving the deglycosylation procedure was successful (Fig.1).

4 Conclusion

UV-curable replica molding in PDMS allows for fast production of large quantities of chips. Click chemistry based polymerization enables work at mild conditions with simple instrumentation.

Results show promising use of thiol-enes in the fabrication of biochemical microreactors thanks to their high diversity and simple surface modification. Surface functionalization can be carried using functional groups already present in the material and can be easily patterned thanks to UV-initiation. Mass spectrometry analysis confirmed effectivity of reactors. Deconvolution of the mass spectra demonstrated a loss of glycan between enzymatically untreated and treated glycoprotein standard.

Acknowledgements

I would like to thank for financial support from Erasmus+ program and Grant Office project (GROFF) CZ.1.07/2.4.00/17.0106. My thanks belong to the colleagues at the Department of Pharmacy, University of Copenhagen, namely Silja Senkbeil for SEM measurements and Kasper D. Rand and Nanna Bøgelund for MS-QTOF measurements.

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P31 POLYMER INCLUSION MEMBRANES OPEN NEW WAYS FOR THE MICROEXTRACTIONS IN-LINE COUPLED TO |CZE

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Summary

Polymer inclusion membranes (PIMs) have several important features, i.e., PIMs are dry and non-porous membranes, which can be prepared ahead of use and stored without noticeable deterioration in extraction performance. In-line coupling of microextractions across PIMs to commercial capillary zone electrophoresis (CZE) instrument has been demonstrated in this contribution. Purpose-made microextraction devices with PIMs were applied, which ensured complete automation of the entire analytical procedure. Practical

applicability of the hyphenated analytical method was demonstrated on direct extraction, injection, CZE separation and quantification of formate (the major metabolite in methanol poisoning) in clinical samples.

1 Introduction

Body fluids are complex samples and their analyses require comprehensive sample pretreatment, which ensures efficient removal of bulk matrix components. A variety of microextraction techniques was developed for this purpose in the past. Supported liquid membrane (SLM) extractions have become very popular and various instrumental set-ups for their in-line coupling to capillary zone electrophoresis (CZE) have been reviewed recently [1]. However, since SLM extractions possess several fundamental drawbacks, such as poor long-time performance and evaporation/toxicity of the solvents [2], application of polymer inclusion membranes (PIMs) constitute an attractive alternative. Transport of ions across PIMs was investigated to a very small degree in the past and further research concerning PIM extractions of small organic ions is of significant importance. Moreover, there are no publications on practical use of PIMs for microextractions in clinical analyses. This manuscript brings an example of a practical use of tailor-made PIM microextraction devices in-line coupled to CZE for rapid analysis of formate in clinical samples.

2 Experimental

An automated Agilent 7100 CZE instrument equipped with capacitively coupled contactless conductivity detector (C^4D) and UV-Vis diode array detector (DAD) was used. Simultaneous C^4D and UV-Vis (at 214 nm) detection of formate was performed for each CZE separation. CZE separations were performed in a fused silica capillary (50 µm ID/375 µm OD, 61 cm total length, 15 and 8.5 cm effective lengths for C^4D and UV-Vis detection, respectively) at +30 kV. Short-end injection was applied for sample introduction from the outlet side of the instrument (50 mbar for 30 s).

The optimized PIMs consisted of 60% (w/w) cellulose triacetate (CTA) and 40% (w/w) Aliquat^{TM} 336. PIMs were in-line coupled to fully automated CZE system (Agilent 7100) using tailor-made microextraction devices according to [3].

Human serum and blood samples were used undiluted, stored at -20°C and allowed to thaw prior to analysis.

3 Results and Discussion

BGE solution consisting of 20 mM L-His and 70 mM acetic acid was selected based on the theoretical prediction and its suitability for rapid CZE separation of formate from other anions in blood samples. Detection sensitivity of C^4D was about an order of magnitude better compared to UV-Vis detection and C^4D detector was chosen as the preferable one. CTA was chosen as the base polymer material in production of PIMs. Aliquat^{TM} 336 was added as anion-exchange carrier and also as plasticizer. Schematic drawing of PIM structure is depicted in Fig. 1. Extraction process and the resulting records demonstrating methanol intoxicating are depicted in Fig. 2

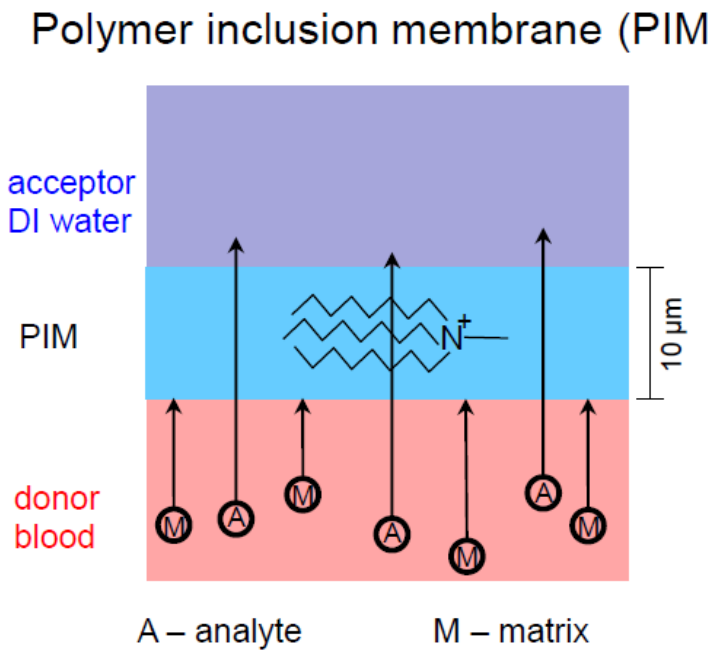

Fig. 1. Schematic drawing of polymer inclusion membrane structure

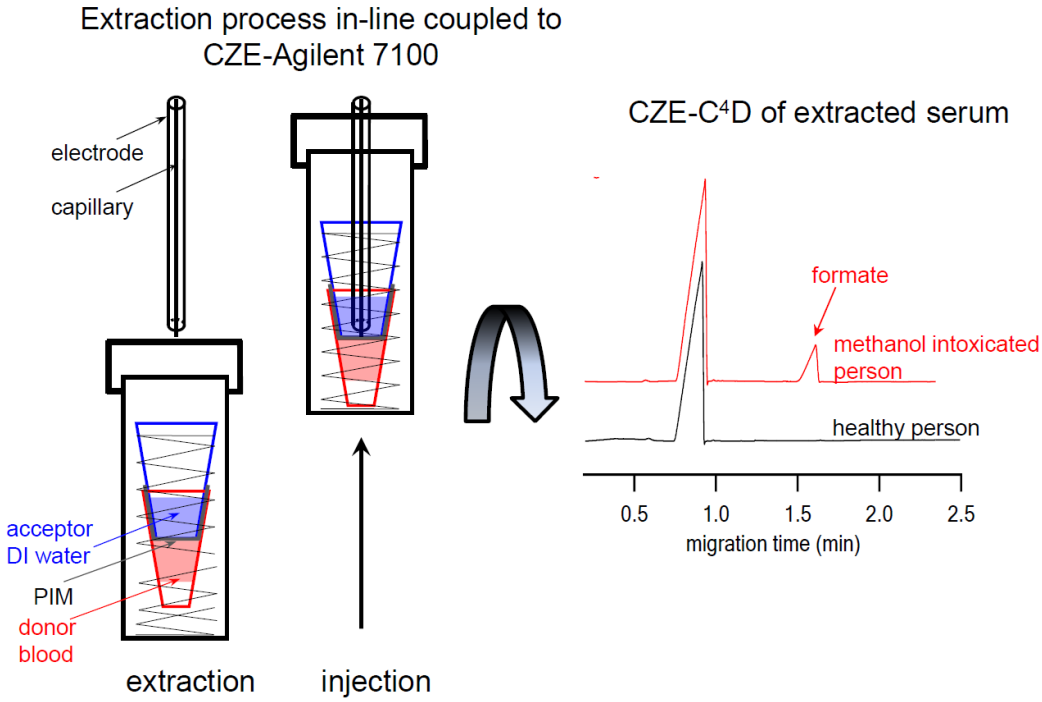

Fig. 2. In-line coupling of microextraction to CZE and the resulting records proving methanol intoxication.

4 Conclusions

In-line coupling of PIM extractions to CZE for determination of formate in clinical samples has been reported. PIMs were produced as ~ 10 µm thin membranes and proved to be sufficiently robust for direct coupling to commercial CZE instrumentation. Tailor-made microextraction devices were used, which accommodated the PIMs and ensured full automation of the entire analytical procedure. Application of pre-assembled, disposable PIM-based microextraction devices compatible with commercial CZE instrumentation might thus be very attractive in clinical analyses.

Acknowledgement

Financial support from the Czech Academy of Sciences (Institute Research Funding RVO:68081715), the Grant Agency of the Czech Republic (Grant No. 13-05762S) and the Regional grant programme of the Czech Academy of Sciences (Project No. R200311404) is gratefully acknowledged. Prof. Robert Bocek (Department of Anaesthesiology, Resuscitation and Intensive Care, Hospital and Policlinics Havířov, Czech Republic) and Dr. Petr Kubáň (CEITEC, Masaryk University, Brno, Czech Republic) are also acknowledged for donation of serum samples of a patient treated with acute methanol poisoning. Dr. Jana Křenková (Institute of Analytical Chemistry of the Czech Academy of Sciences, Brno, Czech Republic) is acknowledged for scanning electron microscopy measurements of resulting PIMs.

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P32 DETERMINATION OF SOME HORMONE ANTAGONISTS BY MICELLAR ELECTROKINETIC CHROMATOGRAPHY

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Summary

Contamination of natural waters is a major concern in many parts of the world. Amongst emerging pollutants, Endocrine Disrupting Compounds (EDCs) appear to be particularly prevalent in the aquatic environment, and some aquatic animals are highly susceptible to their effects. Hence, the aim of our study was to develop a simple and fast method for

separation and determination of five model hormone antagonists, exemestane, toremifene, letrozole, anastrozole, and mifepristone, by micellar electrokinetic chromatography (MEKC). Within the best conditions, 50 mM sodium borate pH 9.5, 50 mM SDS, 10 % 1-propanol, 20 kV, the separation of all the compounds lasts for 7 minutes. The method was successfully applied for analysis of water samples from a local wastewater treatment plant.

1 Introduction

Contamination of natural waters is a major concern in many parts of the world, and there is a limited understanding of the toxicological consequences of pollution of surface waters through discharges of wastewater effluents. Many emerging contaminants originate from human use, and are still present in treated effluents from wastewater treatment plants. Aquatic monitoring is an on-going challenge and a key issue is to identify the most important biologically active compounds currently not covered by existing water-quality regulations. Amongst emerging pollutants, Endocrine Disrupting Compounds (EDCs) appear to be particularly prevalent in the aquatic environment, and some aquatic animals are highly susceptible to their effects as they can be continually exposed to these contaminants and these exposures can be "life-long" [1]. Thus far, the identification of EDCs in aquatic environments has been mostly focused on estrogenic compounds, but recent studies has revealed that the majority of the investigated wastewater treatment plants effluents contained anti-androgenic (AA) as well as estrogenic activity. In addition, the observed feminization of wild fish (roach, Rutilus rutilus) in downstream waters was correlated with exposure to both AA activity and estrogen levels or with AA activity alone [2]. Anti-androgens can bind to the androgen receptor, but are unable to activate it. The structures of chemicals containing androgen receptor antagonist properties can be extremely diverse [3]. Hence, novel, precise and sensitive analytical techniques are the most welcome for identification of anti-androgenic compounds in waste waters [4, 5].

The aim of our study was to develop a simple and fast method for separation and determination of five model hormone antagonists, exemestane, toremifene, letrozole, anastrozole, and mifepristone, by micellar electrokinetic chromatography (MEKC). Separation conditions including pH, concentration and type and concentration of organic solvent as an additive were optimized. The method was then validated and finally it was applied for determination of those compounds in water samples from a local wastewater treatment plant.

2 Experimental

Electrophoretic measurements were performed with the HP 3DCE capillary electrophoresis system equipped with DAD detector (Agilent Technologies, Waldbronn, Germany) using 50 μm I.D. bare fused-silica (FS) capillaries (Polymicro Technologies, Phoenix, USA) with 33.5 cm total length and 25.0 cm effective length. Prior to the first use, FS capillaries were subsequently rinsed with 0.1 M NaOH for 10 min and deionized water for 10 min; between analyses, capillaries were rinsed by 0.1 M NaOH for 2 min,

deionized water for 2 min and by a background electrolyte for 5 min. All the rinsing was done under a pressure of 930 mbar.

3 Results and Discussion

In this study, we focused on the determination of five model hormone antagonists, exemestane, toremifene, letrozole, anastrozole, and mifepristone. Those compounds are low-polar ones with pK_{OW} in the range 2 – 7. Hence, we decided to use micellar electrokinetic chromatography which is ideally suited for separation of non-polar and low-polar compounds. Sodium borate buffer at basic pH with SDS was chosen for the first experiments. First, the effect of pH was studied using 50 mM sodium borate with 50 mM SDS. The best results were obtained at pH 9.5. Then the effect of SDS concentration was evaluated in the range of 25 mM to 100 mM SDS. 50 mM SDS was chosen for the next studies due to its separation performance. Finally, the effect of addition of organic solvent was studied. Methanol, ethanol, 1-propanol, 2-propanol, 1-butanol and acetonitrile were used (concentration of 10 %). The best resolution was obtained with 1-propanol. Then the effect of 1-propanol concentration (1 % - 40 %) was evaluated. The best separation was obtained with concentration of 10 %. Within these conditions (voltage 20 kV), the migration order was as follow: anastrozole, letrozole, exemestane, mifepristone, and toremifene, see Fig. 1.

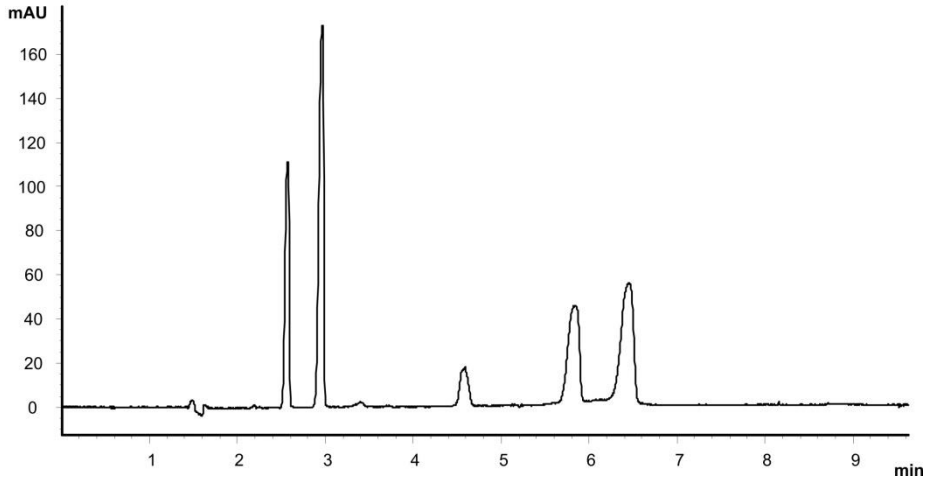

Fig. 1. An example of separation of hormone antagonists by MEKC. BGE: 50 mM sodium borate pH 9.5, 50 mM SDS, 10 % 1-propanol.

After validation of the method, the method was applied for analysis of water samples from a local wastewater treatment plant. The preconcentration step includes SPE extraction through C18 cartridge with a mixture of acetonitrile – water (1:1) as an elution solvent followed by concentration of the eluent under stream of nitrogen and reconstitution in ten times diluted BGE. Those samples were then injected into the capillary electrophoresis instrument. In all the samples, only letrozole was detected in the level of ng/mL.

4 Conclusions

During this work, we developed a simple and fast method for separation and determination of five model hormone antagonists, exemestane, toremifene, letrozole, anastrozole, and mifepristone, using MEKC. Separation conditions were optimized. The method was validated and successfully applied for determination of those compounds in water samples from a local wastewater treatment plant.

Acknowledgement

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P33 LOW-COST 3D PRINTED DEVICE FOR FABRICATION OF nanoESI TIPS BY GRINDING

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Summary

The aim of this work was the development of a simple and low-cost device for nanospray tips fabrication. Although there are several ways of the tip preparation, grinding was chosen as the most suitable in this work. Inexpensive, flexible and reliable method of 3D printing was used for the device construction making it available for any research group with a 3D printer.

1 Introduction

Electrospray ionization is the most frequently used soft ionization technique for on-line coupling of mass spectrometry with separation methods (e.g. capillary electrophoresis or liquid chromatography) [1]. The efficiency of the ionization process is strongly influenced by the quality of the electrospray tip with sharpness, symmetricity and wettability being the key parameters. Generally, the nanospray tips are fabricated from silica fused capillary by three common procedures - pulling, etching and grinding [2]. The grinding is a promising approach in terms of reproducible shape and mechanical robustness. However, it should be noted that thorough optimization of grinding device parameters has to be conducted.

3D printing is currently gaining popularity as an inexpensive fabrication tool with easy adjustment to actual needs [3]. In this work most components were fabricated on the 3D printer to construct a reliable grinding device. A special care was paid to reproducibility of the tip sharpness and maximum symmetricity. Finally, the fabricated tips were used for electrospraying to investigate their mass spectrometric response.

2 Experimental

2.1 Grinding device construction

Based on the previous experience with the nanospray tips grinding we have designed a robust grinding device (Fig. 1) in the user-friendly 3D-modeling software SketchUp (Trimble Navigation, Ltd., USA). The body of the grinding device was fabricated using the FDM printer EASY3DMAKER (AROJA, s. r. o., Czech Republic) from white polylactic acid. The printed parts were assembled with commercially available screws and o-rings. Two small drills (Micromot 50/EF, PROXXON GmbH, Germany) were mounted to control rotation of both the grinding medium and the capillary. The adjustable reel in the center of the body allowed changing of the grinding angle in steps of 5° ranging from 5° to 90°.

2.2 Tip grinding

A bare fused silica capillary (Polymicro Technologies, USA) with 25×375 μm and length of 15 cm was sharpen into the 5° tip by grinding on a sandpaper with the grain size of 1500 and 2000 (Klingspor Abrasive, USA) and polished with fiber-optic lapping film (3M™ Type H - 662XW, 3M Electronics, USA). To assure maximum symmetricity both the capillary and the sandpaper were rotating at 7000 rpm. The quality of the tip was inspected under 45× optical stereomicroscope.

2.3 Mass spectrometry

The MS experiments were conducted on the Velos Pro Dual-Pressure Linear Ion Trap Mass Spectrometer (Thermo Fisher Scientific, Germany) in positive ionization mode. The tip was positioned approximately 3 mm in front of the MS inlet capillary.

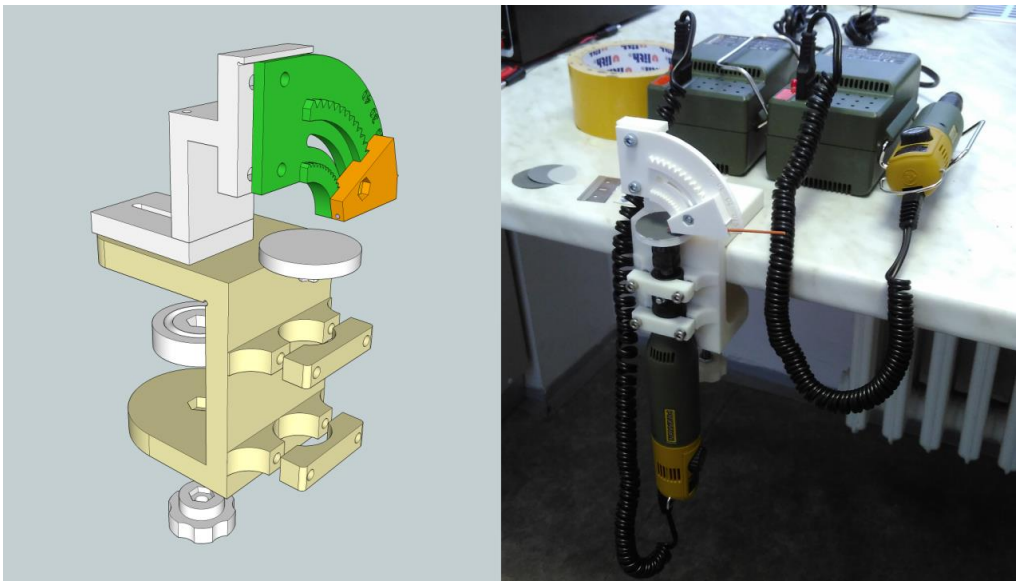

Fig. 1. Scheme of the 3D printed plastic parts (left) and photograph of the assembled grinding device (right).

3 Results and Discussion

Grinding represents a reliable and quick method of nanospray tip fabrication. To obtain symmetrical and sharp tip the isotropic removal of material from the capillary surface must be ensured. For this reason the proposed grinding device includes two microdrilling machines enabling rotation of the capillary and the grinding medium during the grinding process. While rotation of the capillary brings uniformity of sharpening, rotation of grinding medium serves to its surface refreshment.

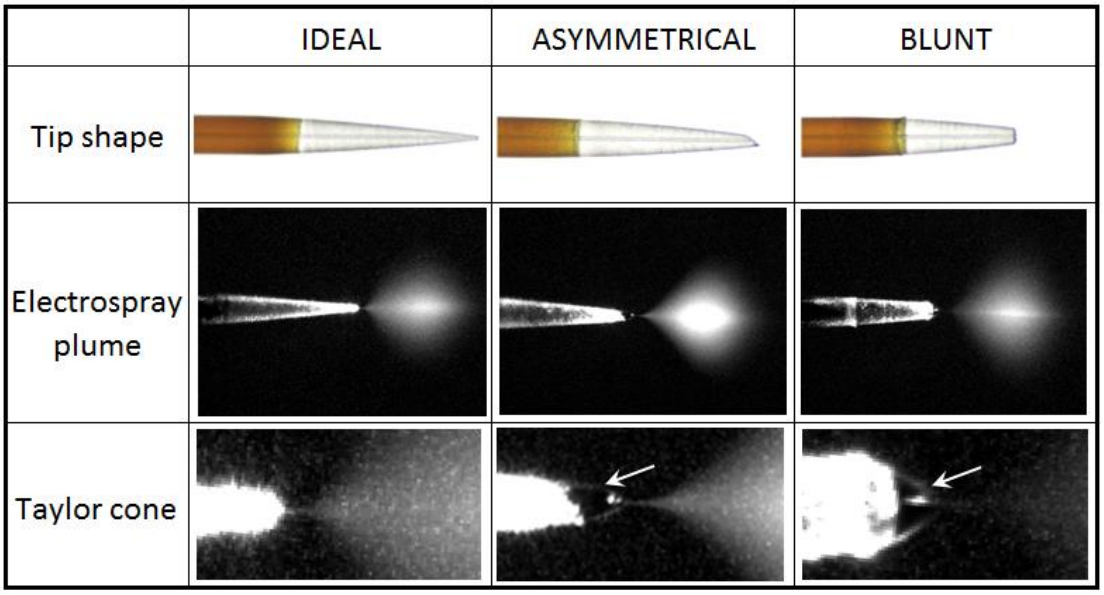

Fig. 2. Pictures of investigated tip shapes fabricated on the developed grinding device and provided electrospray plumes with magnified Taylor cone region (the liquid forming the Taylor cone is labeled by white arrow).

However, the rotational movement is also the source of capillary vibration, which might cause deviation of the capillary end from the surface and give origin to asymmetrical shape. To avoid this phenomenon the capillary end was fixed by PEEK capillary in the closest position above the grinding medium. Under these conditions the device is able to produce tips with good reproducibility of the shape, limited only by the properties (concentricity) of commercially available capillaries.

To investigate the influence of the tip shape on the MS signal, three different tip shapes were fabricated on the developed device. Apart from the ideal shape, the tip with asymmetrical channel and a blunt tip were fabricated too. The tips and corresponding electrospray plumes are depicted in the Fig. 2.

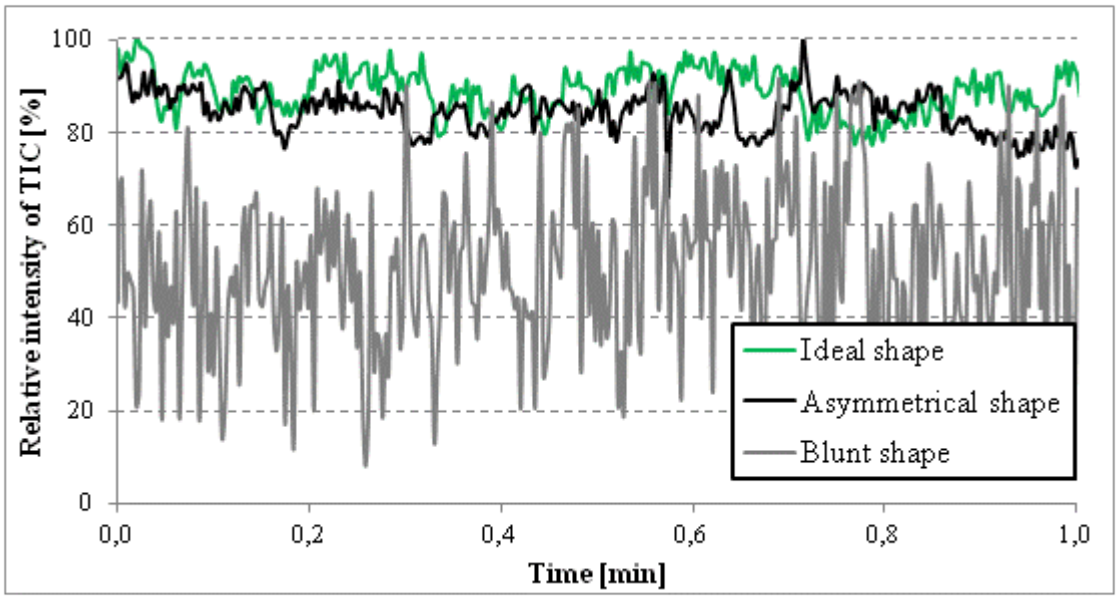

Fig.3. TIC of electrosprayed solution from three different tip shapes. Electrosprayed solution: 0.1% formic acid. Conditions: 5 kV and 90 nL.min^{-1}.

The MS response was observed as TIC stability during electrospraying of solution. Although the electrospray plumes might seem comparable, the fluctuation of signal observed at the blunt tip was remarkable (Fig 3). The instability can be probably attributed to wide base of the Taylor cone. The larger Taylor cone was observed also at the beveled end of the asymmetrical tip. Although in this case the stability of TIC was comparable with the ideally shaped tip, the large volume of the Taylor cone might be problematic due to the memory effect if coupled to an on-line separation method.

4 Conclusions

In this work the device for reproducible capillary tip grinding was designed, printed on the 3D printer and assembled to a working device. This device allows controllable fabrication of tips of different shape, however, only the tips of perfect symmetricity and sharpness provide reliable electrospray. The tips fabricated using the described device

might be useful also for different applications, e.g., electrospray matrix deposition for MALDI, micromanipulation or for preparation of perfectly straight capillary endings.

Acknowledgement
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P34 VOLTAMMETRIC DETERMINATION OF BIOGENIC AMINES IN SAUERKRAUT JUICE

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Summary
The main goal of this work was to develop a simple, sensitive, low-cost and fast square wave voltammetric method (SW) using boron-doped electrode for determination of selected biogenic amines in sauerkraut juice. The limit of detection (LOD) of proposed method was 0.11 μmol/L for histamine, 0.14 μmol/L for tryptamine and 0.49 μmol/L for tyramine. The developed SW method was successfully applied for determination of biogenic amines in several different sauerkraut juice samples with very good recovery (average recovery was 99 %).

1 Introduction
Biogenic amines (BA) are bioactive organic bases formed mainly by the decarboxylation of specific amino acids present in food through the action of bacterial enzymes during the processing, storage or spoilage of food [1, 2]. The total amount and variety of formed amines strongly depend on the present microorganisms. Therefore, BA can be used as indicators of microbial contamination [2]. Bioactive amines may be both essential and harmful for health. When originating from metabolic pathway they are indispensable

compounds for cells either to grow or function in optimal manner. When they are formed by microbial decarboxylation, they are called ‘‘biogenic amines’’ and if they are present in high concentrations, they may induce headaches, heart palpitation, nausea, rash, hypertension and hypotension, and even anaphylactic shock syndrome and death [3]. Biogenic amines occur in different kinds of foods, such as wine, beer, cheese, fruit juices, fish and meat products [4].

For analysis of BA in food samples a number of analytical methods were developed. A dominating method is the reversed-phase high performance liquid chromatography using pre- or post-column derivatization with subsequent UV/VIS or fluorimetric detection [4]. Another used techniques are capillary electrophoresis [5], gas chromatography [6] and various electroanalytical techniques [7], which were used for the determination of biogenic amines in food samples as fish [8], salamis [9], cheeses, beer [10], vine [11], sauerkraut [12], bananas, juices and honey [13], potatoes, spinach [14] or chocolate [15].

The aim of this work was to study electrochemical parameters for the determination of biogenic amines in sauerkraut using boron doped diamond electrode (BDD).

2 Experimental

All electrochemical measurements were performed by Potentiostat/Galvanostat PGSTAT128N (Metrohm Autolab B.V., Utrecht, Netherland), using NOVA ver. 1.10.3 software (Metrohm). A three-electrode system was set in an electrochemical cell with Ag/AgCl/ KCl (3 mol/l) electrode as a reference electrode and a platinum electrode as a counter electrode. As-grown boron doped diamond electrodes 314 (15 000 ppm, 1%) and 321 (8000 ppm, 2%) and glassy carbon electrode (GCE) were used as the working electrode. Cyclic voltammetry (potential range 0 - 1.8 V, potential step 2.5 mV and scan rate 50 mV/s) and square wave voltammetry (potential range 0 – 2,2 V, potential step 0.001 V, amplitude 0.005 V, frequency 70 Hz) were used as electrochemical techniques. As supporting electrolyte a 0.1 mol/l ammonium acetate buffer was used.

HPLC-MS/MS experiments were performed by LCMS-IT-TOF^{TM} (Shimadzu, Kyoto, Japan) analyser with electrospray ionization using LCMS Solution ver. 3.51 software (Shimadzu).

Histamine, tyramine and tryptamine were obtained from Sigma-Aldrich (Steinheim, Germany). All chemicals used in preparation of supporting buffer solutions were analytical grade and were obtained from Sigma Aldrich. Water purified by a Labconco WaterPro PS water purification system (Labconco, Kansas City, MI, USA) was used for the preparation of all solutions.

3 Results and Discussion

First part of our work was focused on the optimization of electrochemical conditions for voltammetric analysis of selected biogenic amines: histamine (HIS), tryptamine (TRYP) and tyramine (TYR). The best current response for HIS and TRYP provides electrode 314 at potentials 1.4 V and 0.9 V respectively and for TYR the electrode 321 at potential 1.5 V. The next step of our work was the optimization of pH of the supporting electrolyte.

Our aim was to develop a voltammetric method compatible with mass spectrometry, therefore the ammonium acetate buffer was chosen as the supporting electrolyte in the pH range 3.5 to 9.5. From obtained data it is clear, that the optimal pH for determination of HIS is 6.5, whereas for TYR and TRYP it is pH 4. For the determination of biogenic amines in real samples the square wave voltammetry was used. The optimized parameters were as follows: potential step (1-5 mV), amplitude (5-25 mV) and frequency (10-90 Hz). Calibration curves for each amine were performed under the optimal conditions. Obtained results were compared with the analysis of biogenic amines on GCE. Here only tryptamine provided a signal, which was unfortunately significantly lower as the signal obtained using the BDD electrode. Additionally, a considerable adsorption of analytes on the surface of GCE was observed, what required its cleaning and polishing. Next, the optimal method was used also for the determination of BA in various samples of juice from sauerkraut. Due to a serious matrix effect, we were able to determine only TRYP. Fig. 1 shows voltammograms for determination of tryptamine in sauerkraut juice. Using the method of standard addition the concentration of TRYP was determined at the level of 76.97±0.64 μmol/l. Obtained results were confirmed by HPLC-MS/MS analysis.

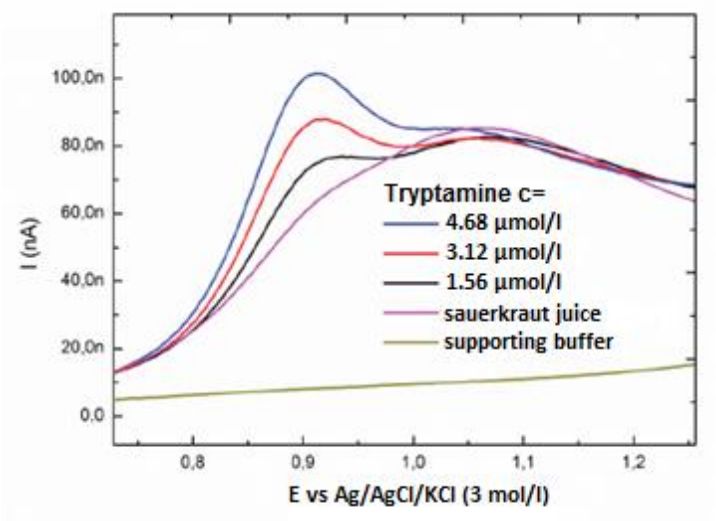

Fig. 1. Square wave voltammograms for determination of TRYP in sauerkraut juice.

4 Conclusions

The aim of our work was to use boron doped diamond electrodes for the determination of biogenic samples in a sauerkraut juice as a traditional Slovak food-stuff. The developed method was successfully applied for the determination of TRYP. We were not able to determine HIS and TYR due to serious matrix effects. The developed method can be used as a base for creation of SMART sensor for simple and fast determination of BA in food samples.

Acknowledgement

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P35 DEVELOPMENT OF ANALYTICAL METHOD FOR SIMULTANEOUS ANALYSIS OF SOBUZOXANE AND ITS METABOLITES/DEGRADATION PRODUCTS

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1 Introduction

Sobuzoxane, (bis(N^1-isobutyloxycarbonyloxymethyl-2,6-dioxopiperazine), Fig.1a), is a novel anticancer drug from the group of inhibitors of topoisomerase II which has been recently approved for the clinical use in Japan [1]. Sobuzoxane has been synthetized as a pro-drug of a bis-dioxopiperazine 1,1‘-ethylendi-3,5-dioxopiperazine (ICRF-154, Fig. 1a) in order to improve its biovailability [2]. Formaldehyde and CO_2 are released during the

esterase-dependent activation of sobuzoxane to ICRF-154. Thereafter, ICRF-154 is likely to be further metabolized in the body to its analog with open bisdioxopiperazine rings 2,2´-{(ethane-1,2-diyl)bis[(2-amino-2-oxoethyl)imino]}diacetic acid (EDTA-diamide) (Fig. 1a) [2].

Thanks to the structural similarity of ICRF-154 and the cardioprotective drug dexrazoxane (Fig.1b), sobuzoxane is also investigated on the experimental animal models as a potential protective agent against antracycline-induced cardiotoxicity. However, the real potential of this drug in this therapeutic indication needs to be further proved.

Although sobuzoxane is a clinically approved drug there is no modern analytical method available for the simultaneous analysis of the parent compound and its active metabolites/degradation products.

Hence this work was focused on the development of a first UHPLC-MS/MS method capable of analyzing sobuzoxane along its metabolites/degradation products (ICRF-154, EDTA-diamide). This method was subsequently utilized for a pilot investigation of the chemical stability of this compound.

2 Experimental

Analyses were performed using Nexera UHPLC system coupled with LCMS-8030 triple quadrupole mass spectrometer with ESI ion source (Shimadzu). During the method development various chromatographic columns – Synergi 4u Polar – RP (3x150 mm or 75 mm, 4 µm, Phenomenex), Hypercarb (3x100 mm, 3 µm, Thermo Scientific), Zorbax SB-AQ (3x150 mm, 3.5 µm or 3x100 mm, 1.8 µm, Agilent) were tested. The mobile phase was composed of a mixture of either water, 0.01% formic acid or 0.5 – 2 mM ammonium formate (A) and methanol or acetonitrile (B) in a gradient mode. 2,2´-{(1-oxoethane-1,2-diyl)bis[(2-amino-2-oxoethyl)imino]}diacetic acid, dexrazoxane and bis(N^1-ethyloxycarbonyloxymethyl-2,6-dioxopiperazine) (Fig. 1b, c) were used as internal standards for EDTA-diamide, ICRF-154 and sobuzoxane, respectively. Quantification was done using SRM for higher sensitivity and selectivity. Linearity of the method was measured for each compound separately and as a mix of all together. Chemical stability of sobuzoxane was tested according to ICH guidelines [3] Q1A and Q1B using acidic (HCl), alkaline (NaOH) and oxidative (H_2O_2) conditions.

3 Results and Discussion

The simultaneous analysis of sobuzoxane, ICRF-154 and EDTA-diamide was complicated from several reasons: 1/ the distinct lipophility of these analytes leading to their completely different retention behavior; 2/ high polarity of EDTA-diamide together with its chelation properties resulted in poor retention, sensitivity and peak shape and 3/ poor solubility of ICRF-154 that required utilization of 100% formic acid in the stock solution which suppressed ionization for polar EDTA-diamide.

Zorbax SB-AQ (3x100 mm, 1.8 µm) was finally selected as a stationary phase as it provides best separation characteristics for this set of the structurally different compounds. Difficulties resulting from iron chelation ability of EDTA-diamide can be overcome by flushing the column with 2mM EDTA solution before the use. EDTA-diamide had to be analyzed separately from ICRF-154 to prevent suppression. Different

internal standards had to be selected for each compound to reflect their dissimilar retention times and structures.

The mobile phase composed of 1 mM ammonium formate (A) and methanol (B) in following gradient: 0-3 min-30% (B); 3-3.5min -30-80% (B); 3.5-7.5 80% (B); 7.5-7.51 min 80-95% (B); 7.51-10 min 95% (B); 10-10.1 min 95-30% (B); 10.1-15 min 30% (B) was chosen as an optimal. Linearity of the method was examined over the concentration range from 100 to 1000 ng/ml for EDTA-diamide and from 50 to 1000 ng/ml for ICRF-154 and sobuzoxane, respectively. A pilot stability study showed that sobuzoxane is more stable in neutral and acidic conditions as compared with alkaline one.

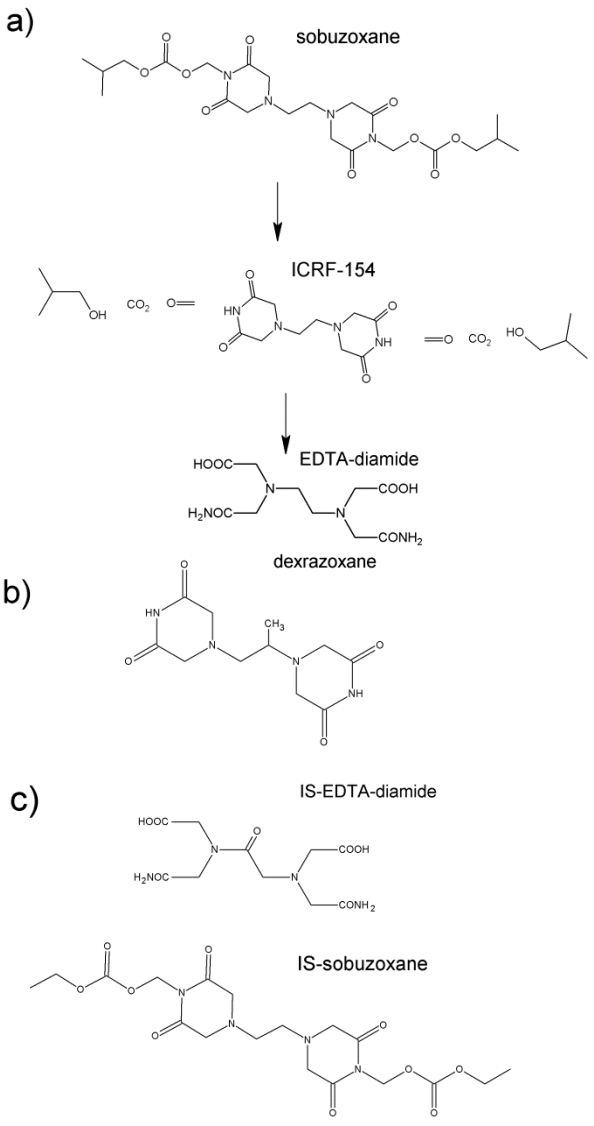

Fig.1. Activation of sobuzoxane to ICRF-154 and further metabolism to EDTA-diamide (a) and chemical structure of dexrazoxane (b) and internal standards (IS) of EDTA-diamide and sobuzoxane (c).

4 Conclusions

The first analytical method for the simultaneous determination of sobuzoxane and its metabolites/degradation products (ICRF-154 and EDTA-diamide) was developed and utilized for a pilot stability study. The method will be further optimized for analyses of these compounds in different relevant biological materials.

Acknowledgement

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P36 EVALUATION OF LIMITS FOR DIRECT NADPH APPLICATION IN ON-LINE CAPPILARY ELECTROPHORETIC METHODS FOR DRUG METABOLISM STUDIES

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Summary

Enzyme assays used for studies of the drug metabolism mediated by cytochrome P450 enzymes (CYP) require presence of not only a corresponding enzyme and a tested drug but also of a sufficient level of NADPH (β-nicotinamide adenine dinucleotide 2'-phosphate reduced). For drug metabolism studies mediated by on-line capillary electrophoretic methods, a direct addition of NADPH seems preferable over commonly used NADPH-regenerating systems because the resulting reaction mixture is simpler and contains lower concentration of substances. On the other hand, NADPH is unstable due to an effect of many factors and the direct addition of NADPH may provide insufficient levels of the cofactor. For these reasons, the stability of NADPH in various incubation

buffers, pH and temperatures, and the effect of NADPH source on CYP reaction were determined in this study.

1 Introduction

Cytochrome P450 enzymes (CYPs) play a key role in biotransformation of more than 75 % of therapeutically important drugs in the human body [1]. Studies of metabolism and the affinity of a candidate compound to this group of enzymes therefore represent a fundamental part of a new drug development process. CYP assays require presence of not only a given enzyme and a tested drug but also of a sufficient level of NADPH serving as a source of electrons within the monooxygenase reaction. In routine protocols, NADPH-regenerating system (NADPH-RS) consisting of glucose-6-phosphate dehydrogenase, glucose-6-phosphate, $NADP^+$ and $MgCl_2$ is commonly used because it provide stable NADPH levels for extended time periods. On the other hand, the resulting reaction mixture is rather complicated, contains high concentration of compounds, and complete separation of reaction products may be challenging. This bottleneck is even more notable in a case of on-line capillary electrophoretic methods where a fused silica capillary is used not only as a separation column but also as a reaction vessel. Since incubation of an enzyme reaction and separation reaction products is integrated into a single analysis, a direct addition of NADPH seems preferable. However, NADPH stability is strongly dependent on pH, temperature and ionic strength, and furthermore reduced nicotine amide cofactors are particularly unstable in phosphate buffers [2]. For these reasons, the main goals of this study was to determine the stability of NADPH in various incubation buffers, pH and temperatures, establish the effect of NADPH source on CYP reaction and formulate recommendations for application of NADPH in drug metabolism studies mediated by on-line CE methods.

2 Experimental

2.1 General CE conditions

A ProteomeLab PA 800 CE System (Beckman Coulter, Fullerton, CA, USA) or Agilent 7100 CE System (Agilent, Waldronn, Germany), both equipped with a photodiode array UV-VIS detector, were used to perform the analyses. The separations were carried out in a 50 µm id, 375 µm od fused-silica capillary (45 cm total length, 8.5 or 10 cm effective length) of Polymicro Technologies (Phoenix, AZ, USA). Before the first analysis of a day, the capillary was rinsed with 0.1 M NaOH for 5 min, bidistilled water for 5 min and BGE for 10 min. The capillary was preconditioned by rinsing with bidistilled water for 3 min and BGE for 5 min before each analysis. An on-column photodiode array UV-VIS detector set to 195 nm was employed for detection of reaction products. Data were collected using the 32 Karat 7.0 (Beckman Coulter) or ChemStation (Agilent) software.

2.2 Determination of NADPH stability

The BGE was composed of 0.1 M potassium phosphate (pH 7.4). The sample was injected by application of 1 psi or 50 mbar (i.e. 6.9 kPa and 5 kPa, respectively) for 4s. Separations were accomplished by concomitant application of 10 kV (-312.5 V/cm,

negative polarity) and a negative pressure of 0.2 psi or 14 mbar (1.4 kPa, small buffer flow towards the cathode) in a capillary thermostated at 25 °C.

2.3 Determination of NADPH source effect on CYP reaction

The BGE was composed of 50 mM TRIS-phosphate buffer (pH 2.5) and 3 % w/v of highly sulfated γ-cyclodextrin as chiral selector. The reaction mixture components were introduced into the capillary as alternate injection of 4 plugs of the solution comprising substrate and NADPH (0.5 psi (3.45 kPa) for 3 s each) and 3 plugs of the CYP3A4 solution (-0.5 psi (3.45 kPa) for 3 s each). The reaction mixture was then formed by diffusion and incubated for 10 min in a capillary thermostated at 37 °C. Concomitant application of -20 kV (-312.5 V/cm, negative polarity) and a positive pressure of 0.2 psi (1.4 kPa, small buffer flow towards the anode) was used for termination of the enzyme reaction and separation of the reaction products.

3 Results and Discussion

First, stability of 1 mM NADPH prepared in 0.5 M K^+ phosphate (pH 7.4), 100 mM K^+ phosphate (pH 7.4), 50 mM TRIS-HCl (pH 7.4), 100 mM Na^+ carbonate (pH 9.4), 50 mM TRIS-MES (pH 8.5), 100 mM Na^+ acetate (pH 4.2), and 18 mM TRIS-phosphate (pH 2.5) and stored at 4, 25 and 37 °C, respectively, was tested. As a result, formation of two major degradation products was detected (data not shown). The first was present in the most of the incubation buffers, higher formation rate was determined at higher temperatures and in the presence of the phosphates. A rapid formation of the second degradation product was observed in acidic conditions, particularly in the acetate and TRIS-phosphate (pH 2.5) buffers.

After it, the effect of NADPH source on ketamine biotransformation was tested. Two series of on-line incubations were carried out, the first with NADPH-RS and the second one with direct addition of NADPH, and reaction products yields were determined. The typical electropherograms are depicted in Fig 1. As can be seen, the upper electropherogram obtained from analysis of reaction mixture containing NADPH-RS is characterized by bulk of $NADP^+$, higher noise and system peaks in the proximity of the reaction products S- and R-norketamine (S-NK,R-NK). The electrophoreogram below obtained from analysis of reaction mixture with direct addition of NADPH at a final concentration of 1 mM is clearer with fully separated peak of substrate S- and R-ketamine (S-K, R-K). No statistically significant differences between production yields of S-NK and R-NK were determined in the tested on-line assays. Higher absolute values of S-NK and R-NK peak areas in the case of NADPH-RS utilization may be caused by different diffusion and/or detection conditions in the solution with significantly higher concentration of ions. The finding of no effect of the reductive equivalent source on the product formation is in agreement with literature data [3].

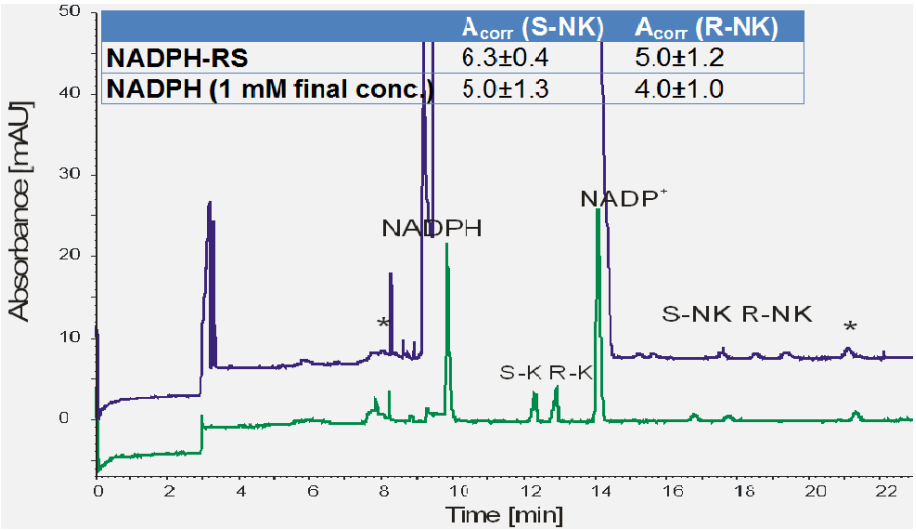

Fig. 1. Typical electropherograms obtained after on-line incubation of CYP3A4, racemic ketamine and NADPH-RS (above) or NADPH (below). S- and R-norketamine (S-NK, R-NK) are products of enantioselective S- and R-ketamine (S-K, R-K) metabolism. * marks peaks corresponding to the CYP 3A4.

4 Conclusions

Stability of NADPH in the incubation buffers commonly used within CYP assays (pH 7.4) is sufficient to provide demanded amount of reductive equivalents even in elevated temperatures. In principle, there is no difference in application of NADPH-RS and direct addition of NADPH. For on-line CE studies of drug metabolism is the latter alternative preferable due to simpler separations and clearer records.

Acknowledgement

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P37 DETERMINATION OF OXIDATIVE STRESS BIOMARKER IN URINE BY ITP-CZE ON A MICROCHIP

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Summary
The main goal of this work was to develop a microchip electrophoresis (MCE) method for the determination of trace concentrations of 3-nitrotyrosine (3-NT), important biomarker of oxidative stress and many inflammatory diseases, in human fluids. We employed an on-line combination of isotachophoresis with capillary zone electrophoresis (ITP-CZE) on a microchip with spectrophotometric detection at 400 nm wavelength. Under the optimized separation conditions a 0.16 μM limit of detection (LOD) (S/N=3) and a 0.52 μM limit of quantification (LOQ) was achieved. The total time of analysis urine samples was less than 600 s. Concentrations of 3-NT in analyzed urine samples were in range 2.0-4.3 μM and the values of relative standard deviation (RSD) did not exceed 5%.

1 Introduction

Oxidative stress is the imbalance between prooxidants and antioxidants in favor of prooxidants, resulting in significant damage to biomolecules as DNA, lipids and proteins. The oxidative damage of proteins is the cause of Atherosclerosis and other inflammatory diseases. 3-NT is one of the most significant biomarker of oxidative stress, therefore the extent of oxidative damage of proteins can be quantified by measuring the 3-NT concentrations in biological samples, such as urine, blood plasma and tissue.

Several analytical methods for trace determination of 3-NT have been developed, such as gas chromatography [1], high-performance liquid chromatography (HPLC) with electrochemical detection [2], HPLC with UV detection [3, 4], capillary electrophoresis [5], ELISA test [6], etc.

Analytical methods with high separation efficiency and sensitive detection technique are needed as the concentration of 3-NT in human fluids is supposed to be very low. MCE has many advantages, such as high separation efficiency, low sample consumption, low running costs, and a wide range of application. One of the significant drawbacks of MCE in trace analysis is a requirement for sensitive detection technique to reach adequate LOD for target analyte. This work was focused on developing an analytical method for the determination of trace concentrations of 3-NT in complex urine samples. ITP-CZE on the microchip with VIS detection technique was used to improve sensitivity of the method.

2 Experimental

Chemicals used for the preparation of electrolyte and model sample solutions were obtained from Sigma-Aldrich (Seelze, Germany). Stock solution of 3-NT was prepared at a 10 mM concentration. Urine samples collected in the morning from 4 volunteers were diluted with deionized water and stored at -30°C. No other sample pretreatment was used before the analysis.

Water demineralized by a Pro-PS water purification system (Labconco, Kansas City, USA) and kept highly demineralized by a circulation in a Simplicity deionization unit (Millipore) was used for the preparation of the electrolyte and sample solutions.

Poly(methylmethacrylate) (PMMA) microchip with coupled separation channels and on-chip conductivity detectors (Merck) were made by technological procedure described in detail in the literature [7]. WellCrom spectrophotometer K-2001 (Knauer, Berlin, Germany) with optical fibers and a 400 nm working wavelength was used for detection of 3-NT.

3 Results and Discussion

Due to pK_a values of 3-NT (pK_{a+1} = 2.02; pK_{a-1} = 6.87; pK_{a-2} = 9.59), the pH of leading electrolyte containing chloride as a leading anion was set to a 8.5. Glycine was chosen as a terminating anion. CZE separations were employed in glycine carrier electrolyte at pH 9.1. The wavelength of spectrophotometric detector was set at a 400 nm due to absorbance maximum of 3-NT (426 nm). The total time of analysis was less than 600 s.

Table 1 represents repeatabilities of quantitative parameters from 3-NT measurements. The model calibration samples of 3-NT for the ITP-CZE separations were in the concentration range 0.2-1 μM. RSD values of migration times were less than 2.3 %. Repeatabilities of peak areas were in range 3.2-6.6%. By using the target method a 156 nM LOD (*S/N=3*) for 3-NT was achieved, and a 515 nM LOQ was evaluated by the equation $LOQ = 3.3 \times LOD$.

Table 1. Repeatabilities of migration time and peak area.

c [μM]	Migration time [s]		Peak area	
	Mean	RSD [%]	Mean	RSD [%]
0.2	594.4	2.3	7.5	6.6
0.5	566.0	1.4	19.2	6.6
1	639.2	1.0	31.2	3.2

ITP-CZE separations of human urine were performed on the microchip in a hydrodynamically closed separation system with suppression of electroosmotic and hydrodynamic flow. Under these working condition, human urine samples I-IV from four different volunteers (29 and 44 years old men and 29 and 27 years old women, respectively) were analyzed. Figure 1 shows the electrophoreograms from the ITP-CZE analyses of 3-NT in urine samples I-IV. The high selectivity was achieved due the use of VIS detection at 400 nm wavelength.

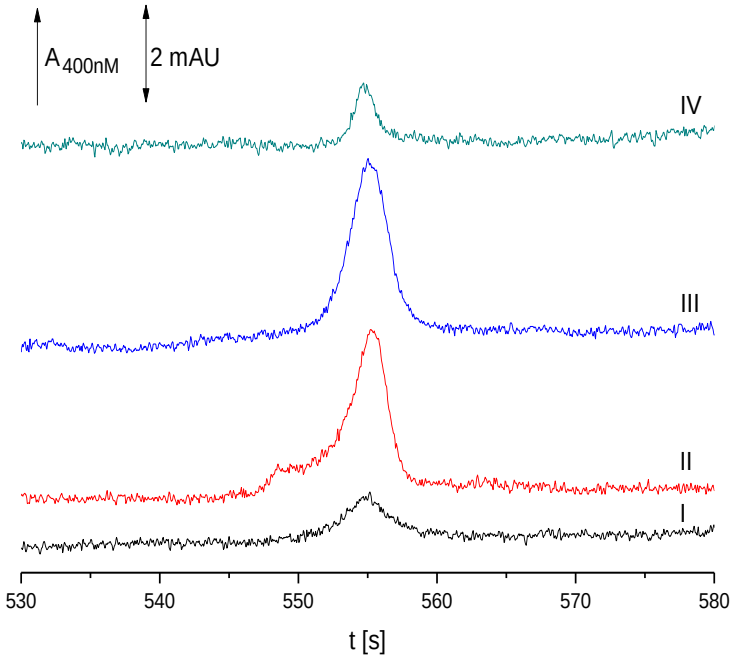

Fig. 1. Electrophoreograms of 3-NT analysis in urine samples I-IV.

Concentrations of 3-NT in urine samples were evaluated from the peak area by a method of standard addition. Three to five repeated ITP-CZE runs were used for data evaluation. RSD values of the peak area of 3-NT in the samples of human urine did not extend 5% (Table 2).

Table 2. Concentration levels of 3-NT in human urine samples.

Urine sample	**I**	**II**	**III**	**IV**
c [μM]	2.6	2.0	4.3	2.2
RSD [%]	4.5	3.7	2.8	4.7

4 Conclusions

The work dealt with the determination of 3-NT in human urine samples of four volunteers using ITP-CZE method on the microchip with VIS detection at 400 nm wavelength. The low LOD (0.16 μM) and LOQ (0.52 μM) values for 3-NT were obtained by the analytical method developed. The RSD values of peak areas and migration times of 3-NT in model calibration samples were in range 3.2-6.6% and 1.0-2.3% respectively. Concentration levels of 3-NT in real samples (2.0-4.3 μM) were evaluated by the method of standard addition with RSD values less than 5%. The total time of analysis did not exceed 10 min. The developed MCE method proved to be highly efficient for determining oxidative stress biomarker contents in human fluids.

Acknowledgement
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P38 CAPILARY ELECTROPHORESIS WITH MASS SPECTROMETRY DETECTION FOR BETA-SECRETASE ENZYME ASSAY

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Summary
In this work we present an optimized method for peptide separation and quantitation by capillary electrophoresis with mass spectrometry detection (CE-MS), which can be used for enzyme kinetics and inhibition assays of β-secretase (BACE). It is a key enzyme involved in creation of amyloid senile plaques, which has been proposed to play essential role in pathogenesis of the Alzheimer's disease. As a result the ongoing medical and pharmacological research is thus focused on the prevention of the production of beta-amyloid by inhibiting of BACE. The Fluorescence Resonance Energy Transfer based assays are used almost exclusively for this purpose, unfortunately these suffer from number of conceptual disadvantages. Employing CE-MS enables high sensitivity, low sample consumption and rapid analysis time, what is more no expensive labelled substrates are necessary.

1 Introduction

Alzheimer's disease (AD) is an irreversible, progressive brain disease that slowly destroys memory and thinking skills. Recent data show that 35 million patients suffer dementia including AD, which is responsible for 50% to 75% of those cases [1]. Consequently, the need to develop drugs to treat AD is a high priority in both academia and the pharmaceutical industry [2].

AD is characterized by many neuropathological changes including neurofibrillary tangles, and loss of synapses and neurons, but it is β-amyloid plaques formation that distinguish AD from other neurodegenerative diseases. This led to the amyloid hypothesis, which is the current leading model of AD pathophysiology. According to this hypothesis, the β-amyloid plaques are generated from transmembrane amyloid beta precursor protein (APP) by their initial cleavage by β-secretase (BACE). In this consequence the BACE inhibition is a promising therapeutic strategy for treating AD and kinetic and inhibition studies of BACE has therefore become key step in such efforts. BACE is an atypical aspartyl protease with acidic pH optimum around 4. Its natural substrate the APP is cleaved between amino acids 671 and 672. APP occurs in number of mutated variants with the so called Swedish mutation having Lys^{670} replaced by Asn^{670} and Met^{671} by Leu^{671} that is faster cleaved by BACE. Hence the model substrates are oligopeptides of usually 8 – 10 amino acids. Most extensively used substrates are fluorescently labels to enable Fluorescence Resonance Energy Transfer based assays, unfortunately these labels significantly lower the substrate solubility in adequate incubation buffers, which makes evaluation of kinetic studies very difficult. Other drawbacks include high background signal, intermolecular quenching and high cost [3]. CE-MS based enzyme assay thus represents a unique alternative with no need of labelled substrates, rapid analysis time and high sensitivity.

2 Experimental

Stock solutions of BACE substrate, [Asn^{670}, Leu^{671}]-APP fragment 667-676, and the pentapeptide product were prepared as 20 mM solutions in dimethylsufoxide (DMSO) and stored at -70 °C. The working samples contained specific amount of substrate and product were prepared in 50 mM sodium acetate (NaAc), pH 4.25 as an incubation buffer (IB), while keeping the total concentration of DMSO at or below 5 %. The sample pH was altered by HCl or NaOH solution to mimic reaction termination. To improve quantitation precision, internal standard human angiotensin II (At II) was added to the sample at the final concentration of 500 nM or 2 µM, respectively. Analyses were performed in an Agilent 7100 CE System (Waldbronn, Germany) connected to a Bruker maXis impact QTOF MS (Bruker Daltonics, Bremen, Germany). A bare fused-silica capillary (45 cm length, 75 µm id, 375 µm od) (PolymicroTechnologies, Phoenix, AZ, USA) thermostated at 25 °C was used for separation. The connection between CE and MS was achieved via the sheath liquid (SL) co-axial ESI interface from Agilent and SL was delivered by an Dionex UltiMate 3000 isocratic pump with a degasser (Thermo Scientific, Dreieich, Germany) via a 1:100 sheath flow splitter. Nitrogen 5.0 (nitrogen of a purity of at least 99.999 %) from a gas cylinder served as the collision gas. Nitrogen for

nebulization of the sample and drying of the formed droplets was delivered by a Genius NM32LA generator (Peak Scientific, UK). Separations were accomplished by application high voltage in positive polarity while keeping electric current below 50 µA. MS detection was performed using ESI in positive mode under the following general conditions: SL methanol-water (1:1, v/v) – flow rate 4 µL·min-1, drying gas flow rate – 5 L·min-1 at drying temperature – 180°C, ESI voltage – 4800 V and nebulization gas – 0.3 bar. The pentapatide product, sequence SEVNL, was detected as $[M+H]^+$ ion m/z = 561.288 and internal standard At II was detected as $[M+2H]^{2+}$ ion m/z = 523.775, the m/z were calculated by the IsotopePattern tool. Quantification was done from extracted ion electropherograms (EIE) with width accuracy ± 0.005 m/z.

3 Results and Discussion

Combination of CE and MS limits, to some extent, buffer composition only to volatile compounds. NH_4Ac and ammonium citrate based BGEs were chosen for the first screening and the IB was based on NaAc. The acetate BGE was preferred due to common anion with IB. Wide ranges of concentration 30 – 70 mM and pH 3.5 – 10.8 of NH_4Ac BGE were tested. Unfortunately no optimal separations were achieved with NH_4Ac as BGE. Subsequently HAc based BGEs were tested and showed high quality separation beyond expectation with optimal concentration of HAc at 12.5 % (Fig. 1).

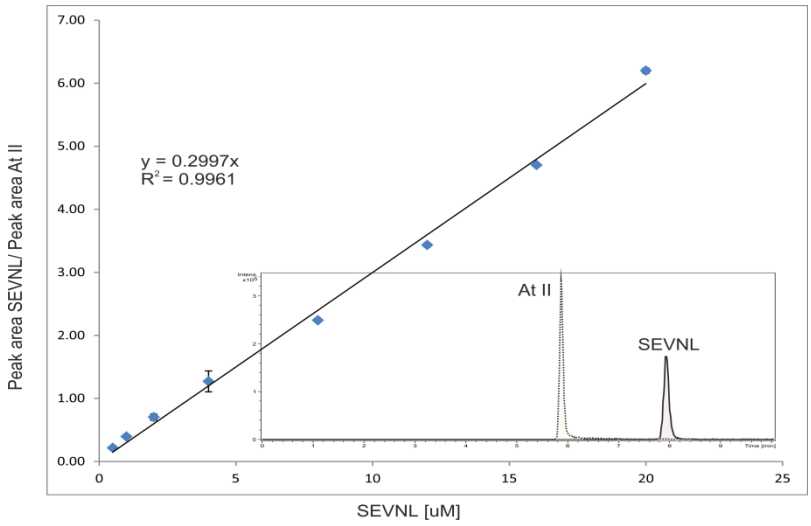

Fig. 1. Calibration curve with sample of extracted ion electropherogram achieved using optimised separation conditions for analysis of sample containing 2 µM internal standard (At II), 2 µM product (SEVNL) and 98 µM substrate (SEVNL/DAEFR).

Calibration curve incorporated in Figure 1 demonstrates high linearity and besides, achieved repeatability of peak area was below 5 % throughout the concentration range needed for kinetic and inhibition studies. These parameters together with LOD as low as 30 nM and LOQ at 100 nM validates this method for further use in BACE enzyme assays.

4 Conclusions

A novel method for quantitative monitoring of no labelled BACE substrate cleavage by CE-MS was developed. Its short analysis time below 10 minutes, miniscule sample consumption and high quality validation parameters enable its employment in kinetic and inhibition studies of BACE, a promising drug target in AD treatment.

Acknowledgement

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P39 HILIC SEPARATION OF AETMA-LABELED GLYCANS ON A WIDE BORE SILICA-BASED MONOLITHIC CAPILLARY COLUMN

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Summary

In this contribution, we report the separation of AETMA-labeled glycans on a wide bore (320-µm i. d.) silica-based monolithic capillary column modified by [2-(methacryloyloxy)ethyl]-dimethyl-(3-sulfopropyl)-ammonium hydroxide.

1 Introduction

Glycoproteins play an important role in various biological functions and thus represent attractive substances for pharmaceutical industry. Their therapeutic activity depends on a specific glycoform and therefore reliable analytical tools and methods are essential for glycosylation monitoring. A coupling of zwitterionic-type hydrophilic interaction chromatography (HILIC) with electrospray-mass spectrometry (ESI-MS) detection has

been recognized as an effective tool for glycan analysis [1]. In this contribution, we report the HILIC-ESI-MS method for analysis of native and labeled glycans utilizing 320-µm i. d. silica-based monolithic capillary column modified by [2-(methacryloyloxy)ethyl]-dimethyl-(3-sulfopropyl)-ammonium hydroxide.

2 Experimental

A 320-µm i. d. silica monolithic capillary column was prepared by acidic hydrolysis of a mixture containing tetramethoxysilane (TMOS), 2-bis(trimethoxysilyl)ethane (BTME), polyethylene glycol, and urea. Then, vinyl groups were introduced to the surface of the silica monolith followed by a thermally initiated grafting of [2-(methacryloyloxy)ethyl] dimethyl-(3-sulfopropyl)ammonium hydroxide (MEDSA) [2].

Glycoproteins (1 mg/ ml) were deglycosylated using a soluble peptide-N-glycosidase F [3]. The labeling was proceeded by reductive amination with (2-aminoethyl)trimethyl-ammonium salt (AETMA). The glycan samples were diluted in 1 ml of weak mobile phase prior injection.

Dionex Ultimate 3000 binary gradient pump with the splitter cartridge for a split ratio of 1:100 (Dionex, Sunnyvale, CA, USA) and the electrically actuated two position injection valve C2-0006EH (VICI Valco Instruments Co. Inc., Houston, TX, USA) with an external sample loop (injected volume 0.7 µl) were used.

Separation conditions were as follows: column temperature 25 °C, flow rate 8 µl/min, mobile phase gradient - solvent A (7/32/1 v/v/v water/acetonitrile/1M ammonium acetate pH 5) for 10 minutes, linear gradient A – B in 30 minutes (B: 15/24/1 v/v/v water/acetonitrile/1M ammonium acetate pH 5). ESI/TOF/MS analysis was performed on Bruker maXis impact (Bruker Corp., Billerica, MA, USA) in the reflectron positive mode.

3 Results and Discussion

A wide bore silica-based monolithic column was synthesized in our laboratory from a mixture containing BTME:TMOS in the molar ratio 1:4. Column was modified to HILIC stationary phase employing MEDSA and its evaluation under the isocratic separation conditions proved its high separation efficiency as 101 000 theoretical plates per meter (calculated for uracil, mobile phase - 95/5 (v/v) acetonitrile/0.1 M ammonium acetate (pH 4.5), flow rate 4 µl/min). Fig. 1 shows gradient separation of labelled glycans enzymatically released from bovine ribonuclease B (a) and human immunoglobulin G (b)

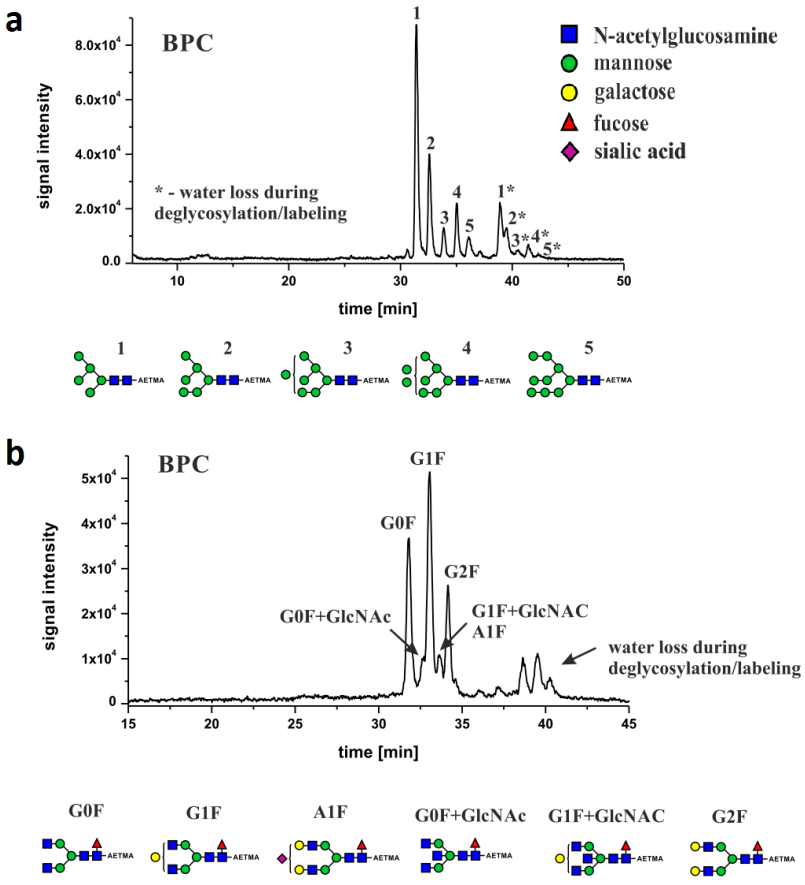

Fig. 1. HILIC-ESI/MS analysis of AETMA-labelled glycans enzymatically released from bovine ribonuclease B (a) and human immunoglobulin G (b).

4 Conclusions

Glycans released from glycoproteins such as ribonuclease B and human immunoglobulin G, having insignificant retention on a reversed phase columns, were labeled by AETMA and effectively retained and separated on the MEDSA modified silica-based monolithic 320-µm i. d. column under the gradient separation conditions and detected by ESI/MS.

Acknowledgement

This work was financially supported by the Grant Agency of the Czech Republic (14-06319S), by the Institute of Analytical Chemistry of the CAS, v. v. i. (RVO68081715), and by the Ministry of the Interior of the Czech Republic (VG20112015021).

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P40 CONSIDERATIONS ON ELECTROLYSIS IN ELECTROMEMBRANE EXTRACTION OF BASIC DRUGS

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Summary
Electrolysis may significantly affect composition and pH values of non-optimized acceptor solutions and have fatal consequences on quantitative EME results for weak and medium strong analytes. Acceptor solutions consisting of high concentrations of weak acids have therefore been proposed as suitable operational solutions for electromembrane extraction (EME) of selected basic drugs. 500 mM formic acid efficiently eliminated the electrolytically produced OH^- ions, offered constant pH and thus long-term EME performance and was easily compatible with subsequent analytical methods. Maximum EME recoveries ranged from 66 to 89% and were constant between 40 and 80 min of EME and no back-extraction of the analytes into donor solutions was observed.

1 Introduction

Electromembrane extraction (EME) is a novel sample preparation technique where target analytes are extracted from an aqueous sample, through an organic solvent immobilized as a supported liquid membrane (SLM) in the pores of a porous hollow fibre, and finally into an aqueous acceptor phase located inside the lumen of the hollow fibre. The driving force for the extraction is an electrical potential sustained over the SLM, with one electrode located in the sample and the other electrode located in the acceptor phase. The electrode reactions take place in the operational solutions during EMEs and may have a direct bearing on changes in composition and pH value of the solutions, especially of acceptor solutions [1,2].

Electrolysis is omnipresent in all electrochemical processes including EME. Electrolysis may significantly affect composition and pH values of operational solutions due to production of H^+ and OH^- ions. In this contribution, aspects of electrolysis in EME were comprehensively considered for a set basic analytes.

2 Experimental

2.1 Electromembrane extraction

Schematic drawing of the EME system and detailed description of the EME procedure were presented in a previous publication [2].

2.2 Capillary electrophoresis

The equipment and conditions for capillary electrophoresis (CE) were also described in detail previously [2]. An Agilent 7100 CE (Agilent Technologies, Waldbronn, Germany) operated at 200 nm and background electrolyte solution consisting of 15 mM NaH_2PO_4 and 15 mM H_3PO_4 at pH 2.23 were used for CE determination of basic drugs.

3 Results and Discussion

Various acidic acceptor solutions (1 mM, 5 mM and 10 mM HCl and 100 mM and 500 mM acetic and formic acid) were tested, which promote EME transfer of basic drugs into acceptor solutions due to their acidic pH. Acceptor solutions consisting of high concentrations of weak organic acids were proven suitable for EMEs of basic drugs since they efficiently eliminated the electrolytically produced OH^- ions, ensured constant pH in acceptor phase and thus constant EME performance. It has been observed that due to the constant pH of acceptor solutions (pH changed by less than 0.2 unit for 500 mM formic acid during 80 min of EME), constant EME efficiencies were achieved at 30 – 40 min of EME and no decrease in extraction recoveries was observed for any longer extraction times. In Figure 1A, a plateau was formed for all recovery curves at 40 min with maximum recoveries ranging from 66 to 89%. Back-extraction of analytes into donor solutions, which is the cause for reduced extraction recoveries and is often reported in non-optimized acceptor solutions [3], was not observed during the entire EME procedure (see Figure 1B). Saturation of acceptor solutions with analytes is often considered as another source of reduced recoveries in EMEs [3] and was thus examined. It has been observed that saturation of acceptor solutions with 30 – 300 µg/mL of basic drugs did not induce decrease in their ERs nor their back-extraction to donor solutions (Figure 2). Nevertheless, the extraction process appeared to be somewhat slower and less efficient for acceptor solutions saturated with 100 and 300 µg/mL of the drugs and the drugs remained partly unextracted for the most saturated acceptor solution even after 80 min of EME.

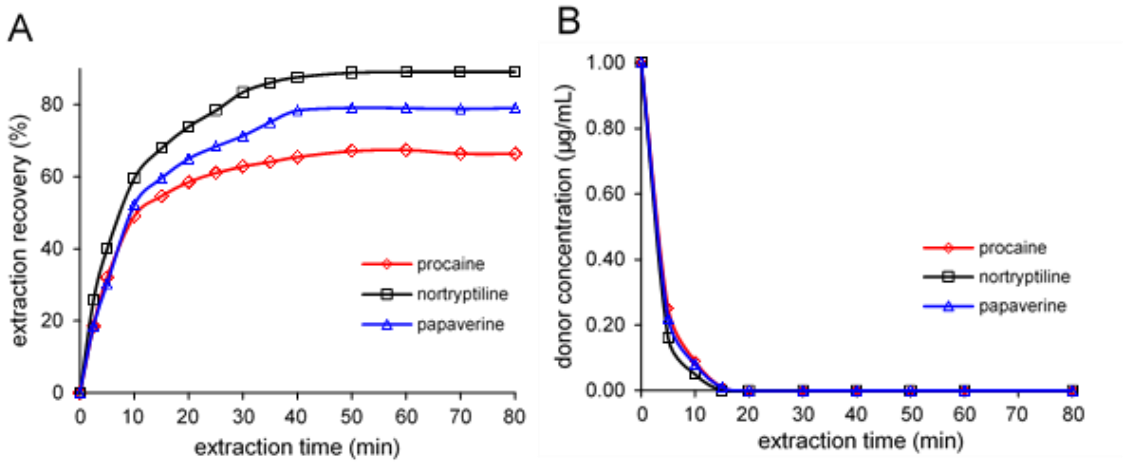

Fig. 1. A. EMEs of basic drugs into optimized acceptor solution (500 mM formic acid). B. Concentrations of basic drugs found in donor solutions after EMEs. EME conditions: extraction voltage, 50 V; extraction time, 0 – 80 min; SLM, ENB; donor solution, basic drugs at 1 µg/mL in 10 mM HCl (pH 1.96); agitation, 700 rpm.

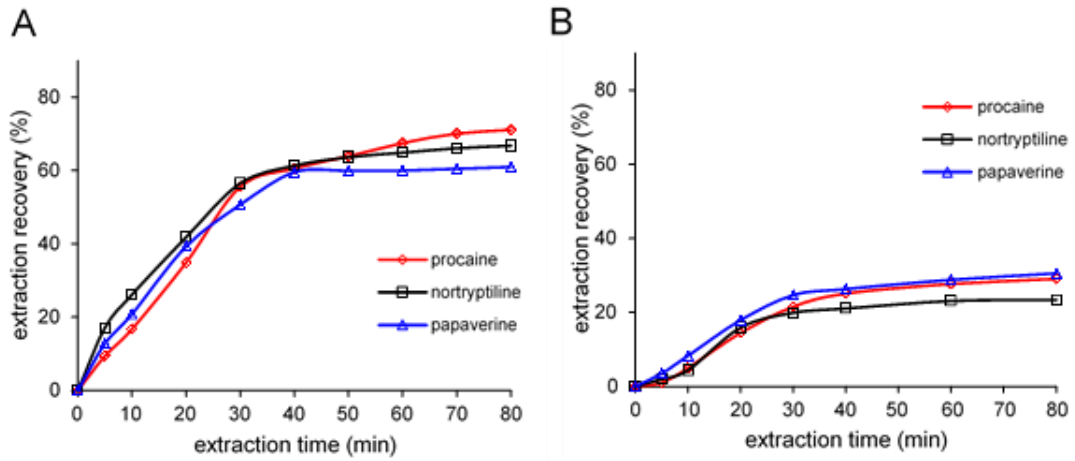

Fig. 2. Effect of acceptor solution saturation with basic drugs on their EME recoveries. A. 500 mM formic acid (pH 1.97) saturated with 30 µg/mL of basic drugs. B. 500 mM formic acid (pH 1.97) saturated with 300 µg/mL of basic drugs. EME conditions as for Fig. 1.

4 Conclusions

The effects of electrolysis can be controlled by proper selection of EME operational conditions. Optimized acceptor solutions ensure constant long-term EME performance due to efficient elimination of electrolysis by-products and once maximum recovery is achieved, the recoveries are constant independently of the extraction time. Saturation of acceptor solutions with analytes has no additional effect on their back-extraction to donor

solutions. The presence of up to 300-fold excess of analytes in acceptor solutions leads only to slightly slower and reduced but stable enrichment of analytes.

Acknowledgement
Financial support from the Czech Academy of Sciences (Institute Research Funding RVO:68081715) and the Grant Agency of the Czech Republic (Grant No. 13-05762S) is gratefully acknowledged.

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P41 DETERMINATION OF CAROTENOIDS IN ALGAES BY SUPERCRITICAL FLUID CHROMATOGRAPHY

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1 Introduction

Carotenoids are naturally occuring tetraterpenes found in various fruits, vegetables, plants algae and bacteria. These are not only essential for human health but also effective as cancer preventing agents, heart attacks and coronary artery diseases. Carotenoids are currently used as food additives as primarily food colorant but also as an antioxidant. Fruits and vegetables are the major contributors of carotenoids in human diet. Indisputably, awareness of microalgae for biofuel, functional foods or as food additive has increased [1, 2, 3].

Food analysis is an area of great importance in determination such compounds. The chromatographic methods are the most widely used ones. Nevertheless, in the last few years the use of supercritical fluids has attracted increasing interest [4, 5]. Carbon dioxide (CO_2) is the most commonly used supercritical fluid, because it is non-toxic, non-explosive, is considered a GRAS (generally recognized as safe) reagent and the experimental conditions required are easily achievable, since critical temperature and pressure are, respectively, 31°C and 73 bar. Supercritical fluid chromatography (SFC) was initially performed with pure CO_2 as the mobile phase, but nowadays SFC is very often carried out in subcritical conditions because CO_2 is modified with an organic modifier or additive in order to increase the solubility of polar compounds [6, 7].

2 Experimental

A method determination of carotenoids in microalgae was developed based on supercritical fluid chromatography for quantitative determination of carotenoids in extracts of *Chlorella, Parachlorella, Scenedesmus quadricauda, Cocomyxa.* The separation of the carotenoids in the acetone extract was performed using two columns Zorbax SB-CN, (4,6 x 75 mm, 3,5 µm particle size) in the series. Liquid CO_2 with addition of methanol (MeOH) as a modificator were used as mobile phase. In order to separate carotenoids in the extract a gradient of co-solvent was used starting with 1% MeOH, kept to 2 min, then increase to 10% in 5 min and kept to 9. min when analysis stopped. The temperature was 40°C, the fow rate was 3mLmin^{-1}, the back pressure was 175 bar and the injection volume was 5 µl. Carotenoid were quantified at 444 nm and identified on the base their retention time and UV-VIS spectra compared with standards.

3 Results

The most viewed carotenoids like beta carotene, lutein and zeaxanthin were determined besides others. Contents of beta carotenes varied from 125 to 300 µg g^{-1} for *Chlorella, Parachlorella* respectively. Contents of lutein were between 235 to 500 µg g^{-1} for *Chlorella, Parachlorella* respectively. Zeaxanthin contents varied between 70 to 106 µg g^{-1} for Chlorella resp. Cocomyxa.

4 Conclusions

The proposed supercritical chromatographic method was used to determine the concentration of carotenoids in algaes and should be seen as a more rapid and environmentally friendly alternative to conventional high-performance liquid chromatography methods utilizing organic solvents.

Acknowledgement

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P42 LIPIDOMIC PROFILE OF PORCINE EPIDERMIS BY MALDI-ORBITRAP MASS SPECTROMETRY USING SHOTGUN APPROACH

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Summary
Lipids of the stratum corneum (SC) are the key components for barrier function of the skin. Unique composition of SC lipid can be disordered in various diseases as well as in ageing process. Number of methods are developed for analysis of specific lipid classes mainly using prior separation. Our study uses shotgun MS approach with MALDI ionization for analysis of porcine epidermis. We have managed to identify more than 100 lipid masses in porcine epidermis.

1 Introduction

The epidermis fulfils an essential ability of the skin, the barrier function which is facilitated by the SC. Although the SC constitutes only 10 % of the skin, it contributes to over 80 % of the cutaneous barrier function [1]. The SC has unique protein and lipid composition. The intercellular lipid mass consist of 50 % ceramides, 25 % cholesterol, 15 % free fatty acids. Lipid precursors either from external source or synthesized by keratinocytes in the stratum granulosum are packed into lamellar bodies and transported along with enzymes to the SC. Lipids are metabolized by enzymes after their release from lamellar bodies finally forming lamellar membranes in the SC [2]. Epidermal lipids represents crucial component of the SC. The barrier function is severely disrupted in number of skin diseases with disordered lipid metabolism [3]. Aged epidermis also exhibit aberrant lipid composition [4]. Various approaches are described for epidermal lipidomics. However, very few uses shotgun approach.

2 Experimental

2.1 Lipid extraction

Skin samples were obtained from the fresh porcine auricles. The samples were heated for 60 seconds at 60 °C and the top layer of the skin, the epidermis was peeled off. Epidermal samples were transferred into a vial and modified Bligh Dyer extraction was performed. Briefly, 2 mL of chloroform/methanol mixture (1:2) were added and samples were then homogenized. After centrifugation, supernatants were transferred into a new vial where 0.5 mL of chloroform and 0.5 mL of water was added. The bottom layer was evaporated under nitrogen and kept under – 20 °C for further analysis. For non-polar lipids modified extraction of Sun et al. was performed. Briefly, 2 mL of hexane, 2 mL of methanol and 0.2 mL of dH_2O was added to the sample and homogenized. After centrifugation, hexane layer was transferred into a new vial. Additional 2 mL of hexane was added to the sample and extraction was repeated. Total 4 mL of hexane were re-extracted with 2 mL of methanol and hexane layer was dried under nitrogen.

2.2 MALDI MS analysis

Lipid extracts were reconstituted with 0.8 mL of chloroform/IPA mixture (1:1) and then mixed with equal amount of 10 $mg.mL^{-1}$ MALDI matrixes: α-cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB) or 9-aminoacridine (AA). Samples were analyzed using MALDI- LTQ Orbitrap XL (Thermo Fisher). Fullscan in positive-ion and negative-ion mode was obtained in the mass range *m/z* 180-2000 using appropriate laser settings depending on the matrix used. Individual masses were fragmented (MS/MS) and analyzed.

3 Results and discussion

Although CHCA and DHB matrixes exhibited similar properties in positive-ion mode, DHB was slightly better, since it provided more fragments in the MS/MS spectra. For this reason, DHB was preferentially used for positive-ion mode and AA for negative-ion mode. We have identified more than 100 *m/z* of which 15 belonged to sphingomyelins (SM), 26 to triacylglycerols (TG), 10 to phosphatidylcholines (PC), 3 to phosphatidylserines, 8 to phosphatidylinositols and 4 to phosphatidic acid and phosphatidylethanolamines. SM were identified and confirmed in positive-ion mode using DHB matrix where they exhibited much greater relative abundance than other lipid classes. TG were identified and confirmed in negative-ion mode using AA matrix with sodium acetate. PC class was selectively identified in positive-ion mode using AA matrix while rest of the glycerophospholipids were identified in positive-ion mode using AA matrix. Also, more than 30 ceramides were identified in positive-ion mode of DHB. However, most of ceramides could not be confirmed due to mass overlap, low relative abundance and low fragmentation potential of ceramides. Moreover, no long-chain ceramides were detected at all [5].

4 Conclusion

Shotgun MALDI-Orbitrap provides rapid lipid profiling of the sample. However, identification of particular species is much more difficult without prior separation technique. More than 100 lipid masses of major intensities were identified in porcine epidermis using MALDI-Orbitrap shotgun approach. Further improvements of the method are required for identification of ceramides and masses with very low relative abundance.

Acknowledgements

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P43 WATER ANALYSIS IMPORTANCE IN ENZYMATIC TRANSESTERIFICATION REACTIONS

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1 Introduction

In natural conditions lipases (triacylglycerols hydrolases, EC 3.1.1.3) catalyze hydrolysis of ester bonds in triacylglycerols. These enzymes are activated on water-oil interface [1]. It is possible to change the direction of reaction by replacing water with some other solvents (organic solvents, ionic liquids) [2]. Nonetheless, water plays important roles in these non-water environments because it not only determines the direction of the reaction (ester bond hydrolysis or synthesis) but also is one of the substrates in the first step of transesterification reaction as well as stabilizes the structure of lipase molecule [3]. For these reasons, this is necessary to optimize the concentration of water in enzymatic transesterification reaction media. The optimal amount of water depends not only on

properties of substrates and solvents but also on the source and form of the biocatalyst [4].

There are two methods commonly used for water analysis in micro-water transesterification reaction media. One of them consists of water activity (a_w) measurements while the second one is Karl-Fischer (KF) titration. The latter method was used in our study since this is more convenient for transesterification processes scale-up.

2 Experimental

2.1 Materials

The following materials were used: sunflower oil (from Kunsagi Eden, Hungary; 0.0214-0.0240% water w/w), 2-methyl butanol (0.318-0.323% water w/w; from Acros Organics). All other chemicals used were of analytical grade.

In situ immobilized lipase (defatted, dried and ground mycelium) from *Mucor circinelloides* strain, owned by the Institute of Technical Biochemistry of TUL (MC preparation), and cube-shaped polyurethane foams (1x1x1cm) overgrown by fungal mycelium and defatted with petroleum ether (MC-PU preparation), were used as biocatalysts in batch and in semi-continuous processes, respectively.

2.2 Water content analysis

Water concentration in the liquid phase was measured by Karl Fischer method using a TitroLine KF titrator (Schott) and a HYDRANAL® Coulomat Oil (34868 Fluka) reagent for coulometric titration.

2.3 Batch and semi-continuous transesterification processes

Batch processes were conducted in 25 ml screw-capped glass flasks in a thermostated shaker (Elpin 374 plus) at 33°C and 220 rpm for 48 h. Portions (0.5 g) of MC preparation were weighed into the flasks and mixed with 10 ml of substrates (oil and alcohol 2:1 g/g (1:5 mol/mol) mixtures), finally the appropriate amount of water was added.

Semi-continuous processes were carried out at 33°C for 48 h in 200 ml thermostated column reactors, each equipped with a peristaltic pump and a container for substrates/products. Each column was packed with 36 g of MC-PU lipase preparation, and 450 ml of substrates mixture was pumped with recirculation (flow rate of 8 ml/min) through the biocatalyst.

3 Results and Discussion

3.1 Influence of initial water concentration on transesterification processes

Batch processes of transesterification reactions were carried out using substrates mixtures with initial water concentration (C_{water}^{o}) ranging from 0.03 to 3.03% w/w. During these processes various changes in water concentration in the liquid phase of reaction mixtures ($\Delta C = C_{water}^{final} - C_{water}^{o}$) that depended on initial water concentration were noticed (Fig 1.).

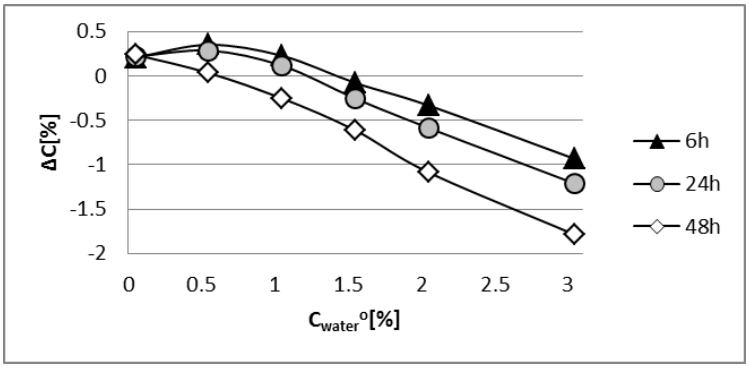

Fig. 1. Water concentration changes in the liqiud phase during transestrification (ΔC) as a function of initial water concentration in substrates mixtures (C_{water}^{o}).

This phenomenon was caused by water molecules migration between liquid phase of reaction mixture and solid phases of the biocatalyst. Noteworthy, the highest yield of esters after 48 h of reaction was obtained for ~0.5% initial water concentration, i.e., when the ΔC value was close to zero (see Fig. 1).

3.2 Changes in water migration rate between the liquid and solid phases caused by DEA

Transesterification reactions were carried out in batch reactors containing mixtures of substrates with the low initial water content (0.03% w/w), and diethylamine (DEA) in concentrations from 10 to 60 mM. After 24 and 48 h of reaction significant changes in the water concentration in the liquid phase, which depended on DEA content (Fig. 2) were noticed. When DEA concentrations were in the range 20-30 mM, the water concentration in the liquid phase (after 24 h and 48 h of reaction) was the lowest while the esters yield was the highest. This phenomenon suggests that polar DEA molecules increase the strength of interactions between water molecules and the enzyme that results in its increased stability as a catalyst.

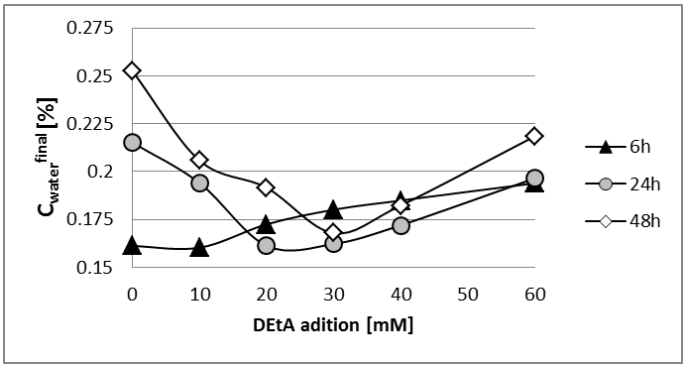

Fig 2. Influence of DEA on final water content in the liquide phase (C_{water}^{final}).

3.3 Lipase operational stability enhancement by water control

Transesterification reaction was carried out in the column reactors in two variants: the 1st - with no water addition to the substrates mixture and the 2nd one - with control of water concentration in the range of 0.35 - 0.40% (previously it was found that at these concentrations there was no migration of water molecules between the solid (MC-PU preparation) and liquid phases). In the first variant, the rapid loss of activity of the biocatalyst occurred (28% of esters yield after 14 days of operation in 7 semi-continuous processes). In contrast, the efficiency of the biocatalyst in the 2nd column was not decreased during 60 days of operation (30 semi-continuous processes of the transesterification reaction), Fig. 3.

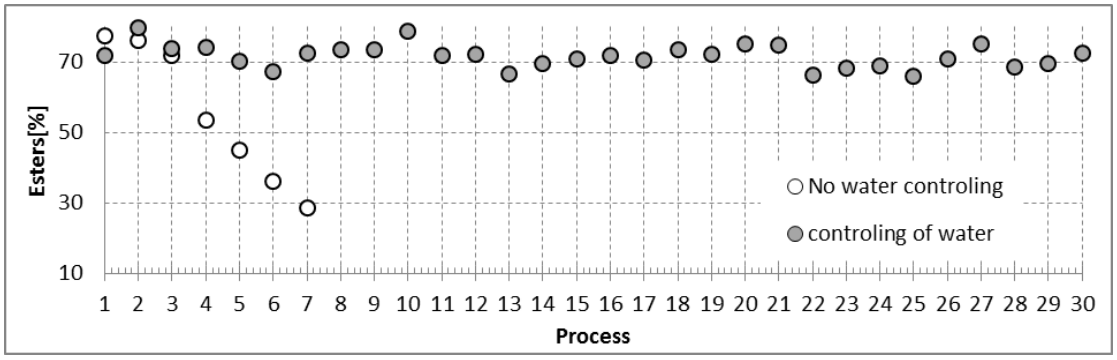

Fig 3. Esters yield in semi-continuous processes carried out with and without water concentration control.

4 Conclusions

Our results prove that measurement of water concentration in the liquid phase by Karl Fischer method and its controlling is required when transesterification reaction is catalyzed by lipase contained in immobilized solid preparations. Adjusting the water concentration to the appropriate level not only increases the yield of esters, but also extends the operational stability of the solid biocatalyst preparations (for up to several months), thereby making processes carried out in column reactors cost-effective.

Acknowledgements

This work was realized within the frame of the project *Biomasa* (No POIG 01.01.02-10-123/09), which was co-financed by the European Union within the European Regional Development Fund (of the Operation Program Innovative Economy 2007-2015).

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P44 DEVELOPMENT OF MICROFLUIDIC TOOLS FOR CELL ANALYSIS

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Summary
In this poster we present our microfabrication approach for fabrication of glass microfluidic devices which we combine with 3D-printed parts in order to develop an easy-to-use instrumentation for single cell analysis.

1 Introduction

In the field of cell-oriented microfluidics a proteomic analysis of single cells using mass spectrometer (MS) still remains a challenging goal. In order to detect rare cells within cell populations we have to be able to differentiate these cells from the regular ones. This approach requires distinct zones of lysate originating from individual cells to be analyzed by MS. Therefore the most important part is sample preparation. Subsequently one can either look for presence of one particular cytosolic protein or fingerprint whole cells one by one.

The most common laboratory-made microfluidic devices are master-molded PDMS devices with certain fragility limiting the researcher in using these devices repeatedly, especially when channels clog during experiments with cells. Options of organic solvents and cleaning solutions (e.g. piranha solution) are limited as well as maximum pressure of liquid that can be applied to flush clogged channels.

Glass as a material overcomes some of the PDMS-related issues. Borosilicate glass devices show high chemical resistance allowing the researcher to use MS-compatible organic solvents during experiments and also harsh cleaning solutions when channels are clogged. In the addition high-pressure flushing does not harm the device. The aim of this work was integration of cell counting based on resistive pulse sensing with electric pulse disruption in a single microfluidic device.

2 Experimental

2.1 System design and fabrication

Graphic patterns of microchannels were created as a bitmap file in Adobe Photoshop software. These patterns were exposed on commercially available borosilicate photomask blank substrates (Nanofilm, USA) covered with 100 nm layer of chrome and 500 nm thick layer of AZ1518 positive photoresist. The exposure was realized using two different

approaches depending on the size of the smallest feature in the created pattern. Structures larger than 20 μm were fabricated using a contact lithography through a mask fabricated using a silver halide high-resolution film and red diode photoplotter FilmStar-PLUS (Bungard Elektronik, Germany). The wafer was then exposed to UV light through this mask. The other approach utilized high-resolution direct laser-writing using μPG 101 Micro-pattern Generator (Heidelberg Instruments, Germany) to fabricate smaller structures down to the limit of 3 μm. Chrome layer was etched for 55 seconds using ammonium cerium (IV) nitrate based etchant and the exposed surface of the borosilicate glass was etched for 25 min in HF/NH_4F buffer at 75°C. Final dimensions of microchannels were 30 x 12 μm. The etched channels were sealed by thermal bonding with borosilicate cover plate at 600°C for 10 hours.

Custom-made plastic frame holding the microfluidic device under the microscope was fabricated using 3D printer based on fused deposition modeling technology (Easy3DMaker, 3D Factories, Germany). The frame contained integrated platinum electrodes and reservoirs for cell suspension, buffers and reagents, all of which were placed outside the microfluidics itself.

2.2 Resistive pulse sensing and cell lysis

For visual control of cells flowing through the sensing gate, the device was placed on the SVM 340 synchronized video microscope (LabSmith, USA). Resistive pulses were monitored as changes in voltage across the sensing gate using Colibrick A/D converter and Clarity Chromatography Data Station (Data Apex, Czech Republic). In order to detect a change in conductivity due to displacement of an electrolyte by cell entering the sensing gate, this gate was designed to have dimensions of 30 x 50 x 12 μm.

After mixing the cell suspension with an organic solvent (e.g. isopropanol, methanol) to facilitate the electric pulse disruption, a drop in the ionic strength of the buffer caused the increase of intensity of the electric field. All our preliminary experiments were performed with 1 μm fluorescent beads and Saccharomyces cerevisiae yeast cells.

3 Results and Discussion

Two micropatterning approaches described in the previous section were tested. The budget-friendly contact lithography was not able to produce sufficiently small and smooth channels for single cell applications mostly due to the lower resolution of the silver halide film. Therefore the best option was direct laser-writing. 3D printing appeared as a flexible and low-cost way of fabrication of precise housing of the microfluidics parts resulting in a plug-and-play system. Special care had to be taken to prevent chipping of glass on the edges of drilled inlet holes which could clog microfluidic channels leaving the device useless. Borosilicate glass microfluidics combined with 3D-printed housing shows good potential for preparation of daily-used miniaturized laboratory systems.

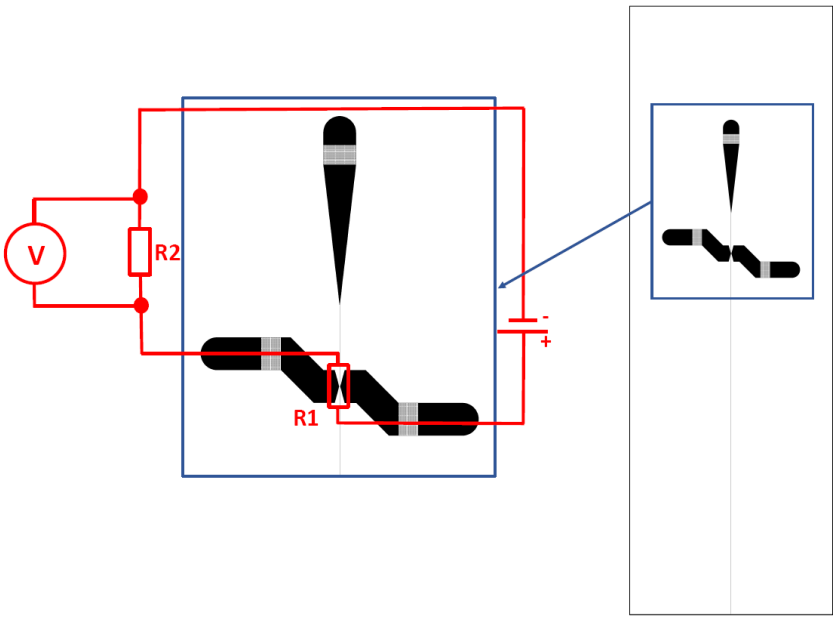

Fig. 1. Design of microfluidic device and representation of sensing circuitry.

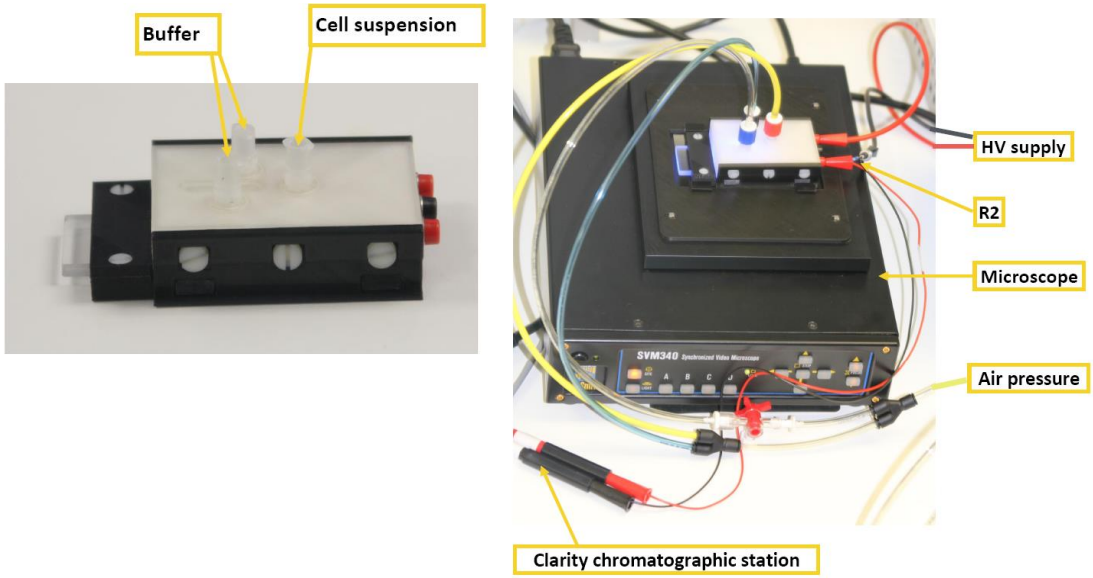

Fig. 2. Microfluidic housing and instrumental set up.

Acknowledgement

Financial support from the Grant Agency of the Czech Republic (14-06319S, P206/12/G014), and the institutional research plan (RVO:68081715) is acknowledged.

P45 OPTIMIZATION OF SHEATH-FLOW CE/MS SEPARATION OF OLIGOSACCHARIDES

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Summary
The aim of this work was to optimize the conditions of the analysis of oligosaccharides using commercial connection of the capillary electrophoresis and mass spectrometry with coaxial sheath-flow interface and electrospray ionization.

1 Introduction

Oligosaccharides belong to group of biologically active compounds with important role in human organism. They are essential as information-carrier molecules indispensable for recognition, trafficking, adhesion and proliferation phenomena. For successful electrophoretic separation, the ionisable functional group should be introduced into their structure as well as chromophore/fluorophore for UV/Vis or fluorescence detection. Aromatic sulfonic acids are widely applied as labelling agents due to their suitable spectral properties and high electrophoretic mobilities almost independent of background electrolyte pH. In this work, CZE separation coupled to mass spectrometry of 8-aminonaphthalene-1,3,6-trisulfonic acid derivatives of oligosaccharides was optimized.

2 Experimental

The maltooligosaccharides (D-(+)-maltose to D-(+)-maltoheptaose), derivatization agent 8-aminonaphtalene-1,3,6-trisulfonic acid (ANTS), ammonium formate, ammonium acetate, acetic acid, ammonia were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium dihydrogen phosphate was from J.T.Baker (Deventer, Netherlands), phosphoric acid from Lachner (Brno, Czech Republic), formic acid from Penta (Chrudim, Czech Republic). The oligosaccharides were derivatized by reductive amination according to the slightly modified procedure described elsewhere [1]. All experiments were carried out using an Agilent 7100 capillary electrophoresis equipped with UV/Vis diode array detector in non-coated fused silica capillary (Agilent, Palo Alto, CA, USA) coupled to ESI source of an AB SCIEX 4500 QTrap mass spectrometer (Framingham, MA, USA) using sheath flow interface.

3 Results and Discussion

In the first part of the work, the separation was optimized using UV detection. Under optimized conditions compatible with MS detection, the seven homologues of ANTS-labelled maltooligosaccharides were successfully separated in sub-minute time range in ammonium formate background electrolyte with pH = 3.0. The composition of sheath flow (ratio of water, methanol and formic acid) and flow rate (in range of 1-5 μL/min)

was tested. Spectra were recorded in enhanced MS mode for increased sensitivity by trapping of ions in first quadrupole of the MS instrument. The influence of the geometry of capillary end in MS source on the stability of the total ion current and on the ionization efficiency of the oligosaccharides was evaluated. The separation in capillary with flat end and separation in capillary with grinded end to sharp angle was compared. The results suggested that exact positioning of the capillary together with the absence of sharp edges has major influence on the stability of a spray and recorded signal.

4 Conclusions

Capillary zone electrophoresis with mass spectrometry was used for the separation of the mixture of ANTS derivatives of oligosaccharides. The effects of the length, diameter and geometry of capillary, composition of background electrolyte and applied voltage on the separation of oligosaccharide derivatives were evaluated together with the influence of ion source voltage, composition and flow rate of sheath liquid.

Acknowledgement

The financial support by the Czech Science Foundation (14-06319S) is gratefully acknowledged.

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P46 PDMS FLUIDIC CHIP FOR MIRNA DETECTION

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Summary

In this work, a polydimethylsiloxane fluidic chip was suggested for isolation and detection of microRNA using magnetic particles and CdTe quantum dots. The fluidic device was equipped with electromagnets, heating element, light source and photomultiplier tube. The microRNA 124 (miR-124) was chosen as a model analyte. It has a connection to prostate cancer, breast cancer, and/or lymphoblastic leukemia.

1 Introduction

Lab on chip is a concept integrating number of sample manipulation procedures (sample preparation, separation and detection) in one single device to speed up the analysis and lower the overall expenses. Currently, numerous polymers have been proven applicable for manufacturing of such devices due to the simple manipulation and repeatability of the manufacturing process [1]. Even though lithographic techniques are often used to manufacture the master for the polymeric chips, currently also 3D printing technology come to the fore offering low costs, affordable instrumentation and resolution sufficient for certain applications. It is not surprising that these types of devices are now often tested for detection of biologically important molecules.

MicroRNAs (miRNA) are small RNAs (~23 nucleotides long), which are involved in posttranslational regulation of gene expression and therefore play important role in many cellular processes such as proliferation, differentiation, apoptosis, etc. [2, 3] . The detection and quantification of miRNAs is very important for the gene expression profiling, however, there are several limitations of miRNAs detection such as their short length and tissue-specific occurrence [4]. Therefore, utilization of powerful tools such as magnetic particles, quantum dots and miniaturized devices may be beneficial in miRNA research.

In this work, the procedure employing magnetic particles for miRNA isolation and QDs for miRNA fluorescent detection integrated in a PDMS-based fluidic system was tested.

2 Experimental

2.1 Fluidic chip manufacturing

The master for the fluidic chip was crafted on Profi 3D maker (3DFactories) by using acrylonitrile-butadiene-styrene using a model designed in computer aided design software Solidworks (Dassault Systèmes SolidWorks Corp.). The fluidic chip itself was made out of polydimethylsiloxane and connected to the syringe pump CMA 40004 (CMA microdialysis AB) to enable the fluid flow. Fluorescence was detected by the photomultiplier tube H10721-20 (Hamamatsu) and the light emitting diode 405 nm (1 W) was used as a light source.

2.2 MicroRNA isolation

miR-124 (5´-UAA GGC ACG CGG UGA AUG CCA-3´) was used as a model analyte. Commercial magnetic particles (Dynabeads oligo$(dT)_{25}$) were used to isolate the miR-124. The signaling probe was labeled with mercaptopropionic acid-capped CdTe QD using a zero-length linker EDC (*N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride).

The hybridization of all components of the isolation and detection complex was done at laboratory temperature for 15 minutes and the analyte was eluted by increasing the temperature to 99 °C for 5 minutes.

3 Results and Discussion

3.1 PDMS-based fluidic analysis platform

Polymeric fluidic devices are elegant and effective tools for fluidic analysis. Moreover, in combination with 3D printing technology, which is providing sufficient resolution for manufacturing of masters for certain applications, they are providing easy to make and easy to change platforms for chemical and biochemical analysis.

Here, we used PDMS to create a device according to the 3D printed master devoted to isolate and detect microRNA (Fig.1). A simple PDMS chip contained two cells (reaction and detection cell) connected by a short channel (2 cm long and 300 μm internal diameter). Two electromagnets were placed on the opposite sides of the reaction cell programmed to oscillate the magnetic field. By this function, it was possible to manipulate the magnetic particles within the reaction cell. A Peltier module with the temperature controller was placed under the reaction cell enabling to elevate the temperature if required by the application of the chip. When the isolation of microRNA from the sample was completed, the final analyte was transferred to the detection cell, which was equipped with a light emitting diode (405 nm) and photomultiplier tube in 90° geometry enabling the fluorescence detection. During the microRNA analysis, green fluorescence (525 nm) CdTe quantum dots were used as fluorescent labels.

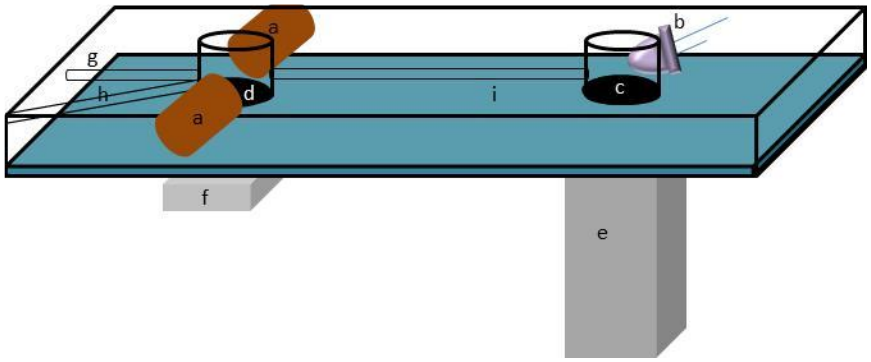

Fig. 1. PDMS-based fluidic analysis platform; a – electromagnets, b – light emitting diode (405 nm), c – detection cell (200 μl), d – reaction cell (200 μl), e – photomultiplier tube, f – heating element, g – mobile phase inlet, h – mobile phase outlet, i – analyte transporting channel.

3.2 Magnetic particle-based isolation with QD-based fluorescence detection

MicroRNA miR-124 (5´-UAA GGC ACG CGG UGA AUG CCA-3´) was used as a model analyte to test the isolation process as well as the fluidic device. The isolation was done using magnetic particles covered by oligoT oligonucleotide. Subsequently, a capture probe (partially complementary to oligoT and partially complementary to miR-124) was hybridized on their surface. The signaling probe consisted of an oligonucleotide complementary to miR-124 labeled by CdTe QD. The final isolation and detection complex is shown in Fig. 2.

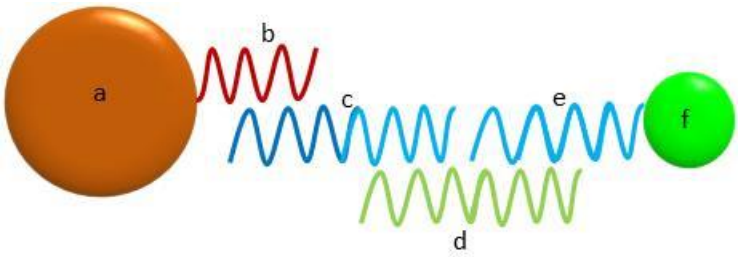

Fig. 2. Scheme of the isolation and detection complex, a – iron oxide magnetic particle, b – oligoT, c - capture probe (oligoA part – dark blue, microRNA complementary part – light blue), d – targeted microRNA, e – signaling probe (microRNA complementary), f – quantum dot.

4 Conclusions

A PDMS fluidic device was designed and manufactured to accommodate the magnetic bead-based microRNA isolation and QD-based fluorescence detection. The isolation process as well as the instrumentation was tested using miR-124 as a model sample.

Acknowledgement

The work has been supported by NanoBioTECell P102/11/1068.

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P47 ELECTROCHEMICAL DETECTION OF MIR-124 ISOLATED BY MAGNETIC PARTICLES

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Summary
MicroRNAs (miRNA) are gaining increasing attention due to their regulatory functions and connection to numerous diseases. In the present study, we used the combination of separation by magnetic particles (MPs) and electrochemical detection for determination of miR-124. The detection was based on hybridization with signaling probe labeled with CdTe quantum dot (QD) covered where QD were detected as electrochemical label using differential pulse voltammetry (DPV).

1 Introduction

MicroRNAs (miRNAs) are a novel class of single-stranded RNAs, which have an important role in the regulation of gene expression at the posttranscriptional level . These relatively short (~23 nucleotides long) RNA molecules are non-coding and play key role in many cellular processes (proliferation, differentiation, apoptosis, etc.) MiRNAs may have a different expression pattern in a patient with a tumour disease in comparison to healthy subjects [1]. The relations between many miRNAs and different types of cancer have been found [2]. One of them is the miR-124, which is highly expressed in neuronal cells and recently its downregulation in several types of human cancer as breast or prostate cancer was reported [3].

Usually is the detection of miRNAs coupled with the usage of a number of bioanalytical methods as are northern blotting, real-time reverse transcription polymerase chain reaction (RT-qPCR), *in situ* hybridization (ISH) and micro-RNA arrays [4]. Opposite these, the electrochemical techniques could be used as suitable analytical tool for nucleic acid determination [5, 6]. Especially utilization of electrochemical labels for the target sequence detection is very widespread [7]. Coupling of electrochemical detection of nucleic acids with the appropriate isolation step using nano- and micro-structured materials (magnetic particles, quantum dots, etc.) is very promising area research.

2 Experimental

The used oligonucleotides (ODNs) synthesized by Sigma-Aldrich have following sequences: 5‘-GCGTGCCTTAAAAAAAAAAAAAAAAAAAAAAAAAA-3‘, 5‘-NH_2-GACCAAGAGGCTCTGGCATTCACC-3‘, 5‘-UAA GGC ACG CGG UGA AUG

CCA-3‘. Electrochemical measurements were performed using AUTOLAB PGS30 Analyzer (EcoChemie, Utrecht, The Netherlands) connected to VA-Stand 663 (Metrohm, Zofingen, Switzerland) in a standard cell with three electrodes. The HMDE was used as the working electrode. An Ag/AgCl/3M KCl electrode as the reference electrode and glassy carbon electrode was used as the auxiliary electrode. As QDs, the CdTe covered mercaptosuccinic acid (MSA) were used. These QDs were prepared according to Tmejova et al. [8]. Next, the conjugation of QD to the signaling probe using N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride and *N*-hydroxysuccinimide was prepared. The procedure of hybridization of MPs (Dynabeads oligo$(dT)_{25}$) with ODN as well as hybridization between target sequences and probes was performed according to Krejcova et al. [9].

3 Results and Discussion

The miRNA isolation and detection complex (Fig. 1) was composed of magnetic particle with coated by oligoT sequence, capture probe containing oligoA part and miR124 complementary part, and signaling probe containing miR124 complementary part and QD.

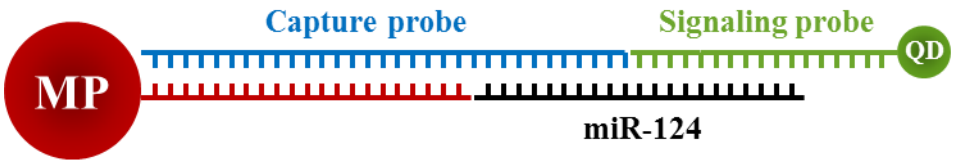

Fig. 1. Scheme of complex.

The first step was the optimization of binding capacity of magnetic particles oligoT. The detection of amount of oligonucleotide unbounded and bounded to magnetic particles was studied using square wave voltammetry (SWV) on the HMDE in acetate buffer (pH 5) (Fig 2A). Then the calibration curve of Cd on the HMDE was performed using DPV in acetate buffer (pH 5) (Fig 2B). Finally the magnetic particles with capture probe about ODN concentration 0.1 μM was used for anchoring of various concentration of miR-124 and then hybridized with signaling probe labeled by QD about ODN concentration 1.7 μM. Electrochemical signal of Cd (– 0.59 V) from quantum dot was subsequently detected by DPV on HMDE in acetate buffer (Fig 2C).

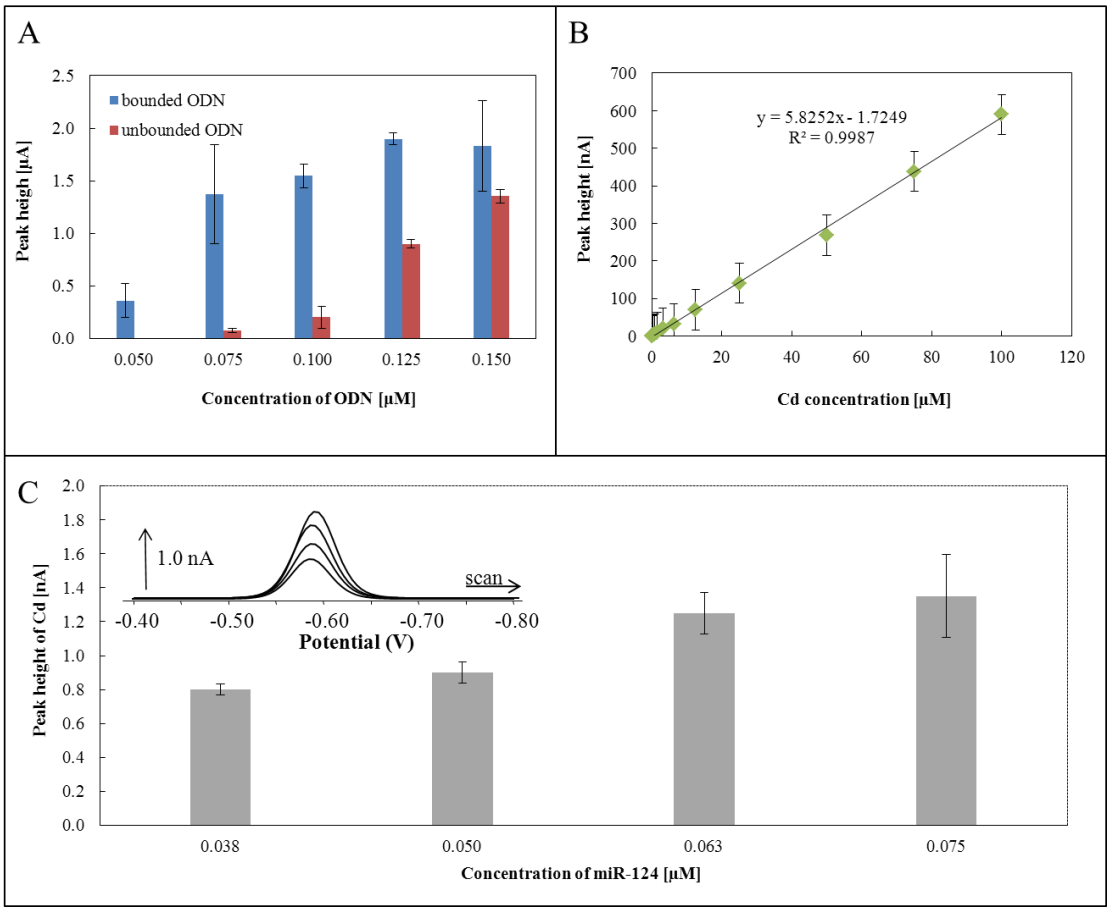

Fig. 2. A) Binding capacity of MPs oligoT. B) Calibration curve of Cd. C) Dependence of peak height of Cd from QD to miR-124 concentration (0.038 - 0.075 µM).

4 Conclusions

The present study offers easy and effective approach for detection of miR-124 which is downregulated in cancer as prostate or breast cancer and thus it could be possible biomarker of these diseases. In the future work we would like to test this separation and detection process in the real samples.

Acknowledgement

The work has been supported by NanoBioTECell P102/11/1068.

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P48 ANALYSIS OF BIOLOGICALLY ACTIVE COMPOUNDS RESEMBLING GROWTH FACTORS OF SOME HERPESVIRUSES BY HIGH-PERFORMANCE SEPARATION TECHNIQUES AND MASS SPECTROMETRY

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Summary
Cells infected with murine herpesvirus produced substances similar to growth factors (MHGF) with transforming effect on non-transformed cells and suppressing the transformed phenotype of transformed cells. To elucidate the structure of MHGF, it was necessary to determine the conditions suitable for isolation of this factor from the culture medium of cells infected with MHV-68 using efficient separation techniques and mass spectrometry. Preliminary results indicate that it could be a substance with a hydrophilic nature, highly probably of zwitterionic type.

1 Introduction

Biological samples are often quite complex and contain molecules that can mask detection of the target molecule, such as in case when the sample exhibits a large dynamic concentration range between the target analyte(s) and other molecules in the sample [1]. Therefore, either more sophisticated combinations of separation and detection techniques or some sample pretreatment technique has to be used [2, 3]. Sample preparation usually

becomes more critical when low detection limits are required, or when potentially interfering substances are present in the samples. Some cells non-productively infected with some herpesviruses produce substances resembling growth factors and showing transforming or transformed phenotype repressing effect on cultured cells [4]. Similar factor (MHGF) has been detected in cells transformed by murine gammaherpesvirus 68, MHV-68 [5]. Studies are now performed to uncover the structure of MHGF.

Nowadays mass spectrometry (MS) is a key analytical technique for identification, quantification, determination of structure, and understanding the physico-chemical properties of the analytes. MS is successfully used in biological, biopharmaceutical, environmental and diagnostic research [6]. Combination of MS with high-performance liquid chromatography with mass spectrometry (HPLC-MS) with electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) is established as the most sensitive and selective analytical technique for the analyses of complex samples [7].

The present work deals with different approaches and analytical aspects on the analysis of biologically active compounds resembling growth factors of herpesvirus.

2 Experimental

All chemicals as used in this work were obtained from Merck (Merck, Darmstadt, Germany), Sigma-Aldrich (Steinheim, Germany) and Fluka (Busch, Switzerland). Formic acid, acetonitrile and water (all LC-MS grade) were purchased from Merck (Merck, Darmstadt, Germany). In this work cell lines (BHK-21, NIH-3T3 and HeLa cells) and virus (MHV-68, MHV-76) were used. The transforming and transformation repressing activity was titrated in BHK-21 cells or in Hela cells, respectively.

Fractionation procedure was performed on the RP-HPLC Separon SGX C18 column (25 fractions were obtained) and on FPLC Sephadex G15 column (after using phosphate buffer with pH=7.2 for elution 7 fractions were obtained, while 9 fractions were obtained when the same sample was eluted with deionized water). All obtained fractions were tested for biological activity.

HPLC-MS experiments were performed on instrument LCMS-IT-TOF™ analyzer (Shimadzu, Kyoto, Japan) equipped with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), respectively. HPLC experiments were performed on Ascentis C18 column (100/2.1 mm; 5 µm, Sigma-Aldrich) using a gradient elution 10-90% (water – acetonitrile) with a 0.2 ml/min flow rate. The column was thermostated to 40°C. Data acquisition for MS-MS3 experiments was performed in range 100-1000 m/z under following conditions: positive and negative ionisation mode (+ 4.5 kV / - 3.5 kV), drying gas flow rate (10 L/min), drying gas temperature (200°C). Fragmentation of selected ions was performed using 25% energy and 25 % collision gas with fragmentation window of ±1.0 m/z. For data acquisition and evaluation LCMS Solution ver. 3.51 (Shimadzu) software was used. The chemometric evaluation was realized by MSXelerator software ver. 2.4 (MSMetrix, Maarssen, Netherlands).

3 Results and Discussion

To elucidate the structure of biologically active compounds resembling growth factors of selected herpesvirus, it was necessary to determine the conditions suitable for isolation of

this factor from the medium of infected with murine herpes virus using high-performance separation techniques. The first step was the fractionation of samples obtained from the medium of cells infected with MHV-68 and MHV-76 using of FPLC Sephadex G15 column and RP-HPLC Separon SGX C18 column. Each obtained fraction was tested for biological activity and as well as analyzed by HPLC-IT-TOF MS analyzer with ESI and APCI ionization in positive and negative ionization modes (differences between fractions with and without biological active is shown on Fig. 1). We have discovered from the obtained chromatograms and MS spectra that the individual fractions have a different composition.

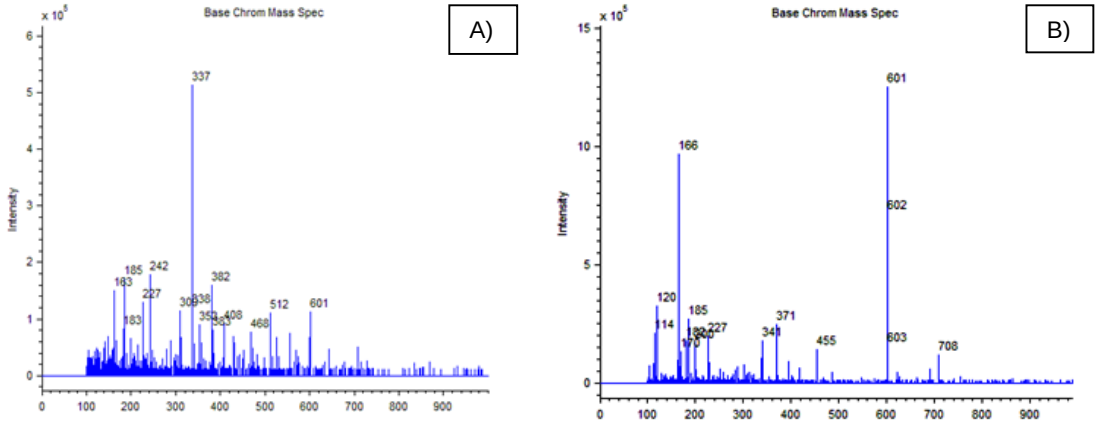

Fig. 1. MS spectra from HPLC-IT-TOF-MS analyses of fractions with (A) and without biological activity (B) obtained by the FPLC fractionation procedure on the Sephadex G15 column after elution by deionized water.

The fraction obtained by the FPLC separation with phosphate buffer reporting highest biological activity was used in experiments with solid phase extraction (SPE). Because of unknown structure of biologically active substances induced murine herpes virus, different types of SPE sorbents at different pH were tested. Based on results obtained from SPE it is possible to conclude that biologically active compounds induced by murine herpes virus contain in their molecule phenolic structure (they are ionized at higher pH 9 and pH 11) and therefore they are not retained on the C8 column. On the other hand, they are retaining on the propylamine column (weak annex), as well.

4 Conclusions

The present work deals with different approaches and analytical aspects on the analysis of biologically active compounds resembling growth factors of herpesvirus MHV-68 and MHV-76. For this purpose the sample pretreatment techniques, multidimensional separations and mass spectrometry were successfully used.

Further experiments exploring the nature, structure, molecular and other biological properties of hitherto unknown compounds are now in the progress.

Acknowledgement
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P49 DERIVATIZATION STUDY OF SELECTED STEROIDS FOR LC-MS ANALYSIS

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Summary
For the purposes of trace analysis of 1,4-androstadiene-3-one-17β-ol (Boldenone) and 17-β-estradiol in water, waste water, soil and for the further phytosterols transformation studies dansylation reaction was studied using dansylchloride with different catalysts as derivatization reagents. For 1,4-androstadiene-3,17-dione (ADD) and 1,4-androstadiene-3-one-17β-ol (Boldenone) oximation reaction using *O*-phenylhydroxylamine hydrochloride reagent was also evaluated.

1 Introduction

There are increasing numbers of steroid hormones applications in the field of human and animal medicines. Considerable part of the steroid hormones belong to the endocrine disrupting chemicals which bio accumulate in the environment. They are harmful to organisms even at the concentration level less than 1 ng/L. PNEC value (Predicted No Effect Concentration) for 17-β-estradiol (E2) is 1 ng/L (3.67 pM) [1].

Practical trace analysis is generally in favour of GC-MS but steroid hormones must be transformed into corresponding derivatives, which can be difficult since steroid hormones contain polar keto and hydroxyl functional groups. Despite the versatility of LC-MS, ionization efficiency varied between different ionization techniques and compounds. Ionization of estrogens by ESI and APCI were less efficient than that of more polar compounds. Chemical derivatization using appropriate derivatizing reagents could improve ionization and enhance signals in LC-MS and the ionization possibilities of poorly or nonionizable analytes in liquid chromatography-mass spectrometry. The conversion of carbonyl groups to hydrazones with quaternary ammonium and pyridinium groups or oximes and derivatization of hydroxyl groups with charged groups or easily-ionizable groups that usually contain tertiary nitrogen atoms are the main strategies applied for the detection of anabolic steroids in positive ESI mode [2, 3]. Dansyl chloride has been widely used as a derivatizing reagent for fluorescence detection and for MS detection of phenols and amines, but not for general alcohols. Recently, two procedures for dansylation of non phenolic (unactivated) alcohols using selected basic catalysts have appeared. By the first procedure the dansylated derivative was formed after incubation of the test compound and excess of dansyl chloride in dichloromethane in the presence of 4-(dimethylamino)-pyridine (DMAP) and N,N-diisopropylethylamine at 65 °C for 1 h, with an overall yield of 96% [4]. In the second procedure only DMAP in acetonitrile was used for derivatization [5]. Therefore, the main goals of our study were to a) test dansylchloride reagent with the addition of different basic catalysts including 1-ethyl-3-methylimidazolium acetate as a representative of ionic liquids b) find optimal conditions for the reaction and c) evaluate the stability of the final derivatives during storage. We have selected two compounds containing hydroxy group for our study: the first having one keto group and one (unactivated) hydroxy group (Boldenone) and second one having two different hydroxy groups (17-β-estradiol). The latter was chosen for comparison of the hydroxyl group reactivity (phenolic versus unactivated). The second goal was testing oximation reaction using *O*-phenylhydroxylamine hydrochloride reagent with boldenone and ADD.

2 Experimental

Stock solutions (30 µg/mL) of 17-ß-estradiol, Boldenone and ADD in methanol (17-ß-estradiol and ADD) and in toluene (Boldenone) was used for all experiments. For optimizing the derivatization process, the selected reagents (catalysts) given in the Table 1 were tested for their ability to derivatize the compounds of interest. Derivatization was conducted as follows: 30 µL of sample solution was evaporated with a stream of nitrogen and afterwards redissolved with 100 µL of each reagent. The mixtures were kept at 60 °C, 10 min or at 65°C for 60 minutes. Subsequently, they were evaporated and redissolved in

1000 µL of acetonitrile prior to LC-MS analyses. The temperature and reaction time were set based on the literature.

Table 1. Overview of dansylation experiments and experimental conditions.

Experiment No.	Steroid	Reagents		Reaction
1	17-β-estradiol	Dansylchloride (1 mg/mL) (DSC) (old) 100 mM $NaHCO_3$		Dansylation 60 °C, 10 min.
2	17-β-estradiol	DSC (1 mg/mL) (fresh) 100 mM $NaHCO_3$		Dansylation 60 °C, 10 min.
3	17-β-estradiol	DSC (1 mg/mL) (fresh) 100 mM $NaHCO_3$, pH 10.5		Dansylation 60 °C, 10 min.
4	17-β-estradiol	DSC (10 mg/mL), N,N-diisopropylethylamine, 4-(dimethylamino)pyridine (DMAP)	DCM	Dansylation 65 °C, 60 min., after 10 min vortex
5	Boldenone	DSC (10 mg/mL), N,N-diisopropylethylamine, DMAP	DCM	
6	17-β-estradiol, Boldenone	DSC (10 mg/mL), DMAP	ACN	
7	17-β-estradiol, Boldenone	DSC (10 mg/mL), N,N-diisopropylethylamine, DMAP	ACN	
8	17-β-estradiol, Boldenone	DSC (10 mg/mL), 1-ethyl-3-methylimidazolium acetate	ACN	

For the oximation reaction 30 µl of ADD and Boldenone solution was evaporated with a stream of nitrogen at 40°C, 125 µl of *O*-phenylhydroxylamine hydrochloride solution in dry pyridine (105 mg in 2 ml) was added, and the oximation mixture was kept at 70 °C for 30 min, then the mixture was evaporated with a stream of nitrogen and redissolved in 1000 µL of acetonitrile prior to LC-MS analyses.

LC-MS measurement was performed using an LCQ Accela Fleet instrument (Thermo Fisher Scientific, San Jose, CA, USA) equipped with electrospray (ESI), atmospheric pressure chemical (APCI), and atmospheric pressure photo (APPI) ionization sources and a photodiode array detector. A 3 µm, 150 mm × 2 mm, Luna C18(2) column (Phenomenex, Torrance, CA, USA) was used with water-acetonitrile-formic acid mobile phase. Mobile phase A used 5% of acetonitrile + 0.1% of formic acid; mobile phase B used water; mobile phase C used 100% of acetonitrile + 0.1% of formic acid (in vol.%).

The gradient was: 0 - 1 min (70 % A, 2 % B, 28 % C), 1 – 15 min (70 % A - 5 % A, 2 % B, 28 % C – 93 %). Injection volume was 10 µL and flow rate 0.250 mL/min. APCI capillary temperature was 275 °C, APCI vaporizer temperature 400 °C, sheath gas flow 58 L/min, auxiliary gas flow 10 L/min, source voltage 6 kV, source current 5 µA, and capillary voltage 10 V.

3 Results and Discussion

The first part of this study was focused on finding an optimum derivatization procedure for the three investigated compounds. Based on our preliminary experiments we finally decided to follow the most cited literature data regarding the temperature and time (60 °C, 10 min. for dansylation of 17-β-estradiol, 65 °C, 10 min. for dansylation of Boldenone). The yield of dansylation reaction of 17-β-estradiol is almost quantitative in all cases because of presence of phenolic group in the molecule of 17-β-estradiol. The reaction is taking place very easily even in the water solution of hydrogen sodium bicarbonate. Dansylation of steroid compound containing both keto and hydroxy groups (Boldenone) was not tested using water solution of hydrogen sodium bicarbonate, because of the presence of unactivated hydroxy group in the molecule of Boldenone. Therefore we have studied the described procedure using N,N-diisopropylethylamine and 4-(dimethylamino)pyridine (DMAP) as dansylation catalyst. Despite of the catalysts presence in the reaction mixture and in the contrast to published data [4, 5] only one third of Boldenone has reacted yielding dansyl derivative. When we have studied this derivatization procedure using N,N-diisopropylethylamine and 4-(dimethylamino)pyridine (DMAP) as dansylation catalyst and 17-β-estradiol as a substrate there was ca. one third of dansyl derivatives present as didansyl derivatives (see Fig. 1).

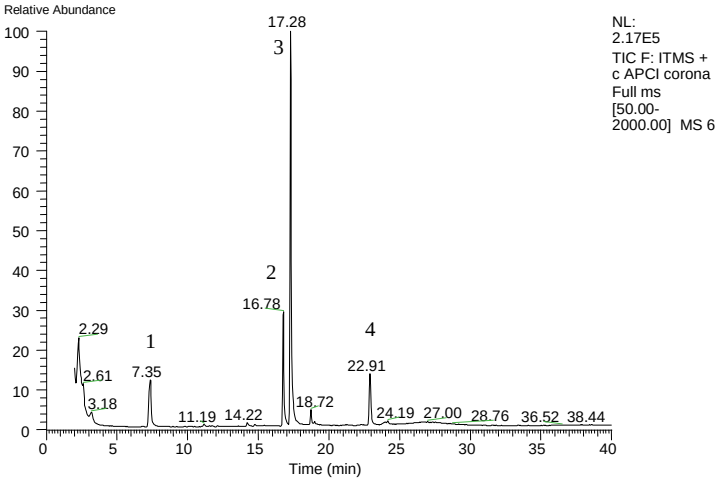

Fig. 1. Reaction mixture after dansylation of Boldenone and 17-β-estradiol. 1 - unreacted Boldenone, 2 - dansylated Boldenone, 3 - dansylated 17-β-estradiol, 4 - didansylated 17-β-estradiol.

Finally, the stability of dansylated-derivatives over six month was studied. During the whole experiment, we didn´t find any significant difference between samples stored in the fridge and freshly prepared samples for none of these steroid compounds. However, some impurities in the Boldenone dansylation mixture over time were detected. In contrary to dansylation reaction the oximation reaction of Boldenone and ADD proceeds smoothly and quantitative yielding typical pairs of geometrical isomers – see Fig. 2. To the best of our knowledge, oximation reaction of Boldenone and ADD with *O*-phenylhydroxylamine hydrochloride is reported here for the first time and the reaction products can be used as a suitable derivatives for the determination of both compounds.

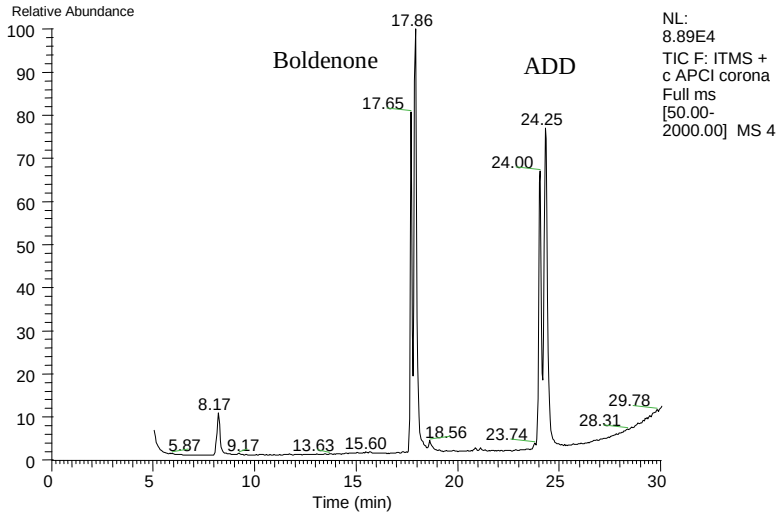

Fig. 2. Oximation reaction of Boldenone and ADD with *O*-phenylhydroxylamine hydrochloride.

4 Conclusions

Study of dansylation reaction revealed that it is possible to use this reaction for the simple and quick determination of 17-β-estradiol, even in 100 mM water solution of hydrogen sodium bicarbonate. Only one third of Boldenone having unactivated hydroxy group reacts with dansylchloride in the presence of N,N-diisopropylethylamine and 4-(dimethylamino)pyridine. ADD reacts smoothly and quantitative with *O*-phenylhydroxylamine hydrochloride.

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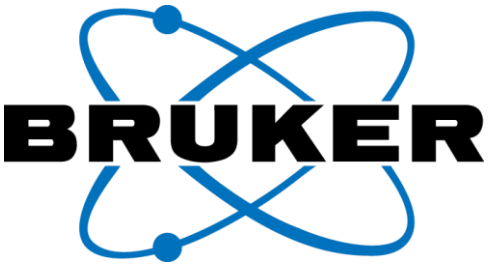

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