CECE 2014

11th 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 2014. With this 11th CECE in a row we are entering the second decade of the conference. As in the previous year we start with lectures by young scientists (CECE Junior), followed by two days of invited lectures and poster sessions. This book of proceedings includes the program of all three days. This year the meeting is free of charge for all perticipants thanks to the financial support by the European Social Fund and the state budget of the Czech Republic (CZ.1.07/2.3.00/20.0182). Of course our original 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" remains intact. The organizers want to thank the invited speakers and all the participants and hope that you will enjoy the scientific presentations as well as personal contacts and informal discussions.

Brno, October 18, 2014

The CECE 2014 conference is dedicated to Prof. Jaroslav Janák, the founder of the Institute of Analytical Chemistry, on the occasion of his 90th birthday

Jaroslav Janák was born in Užhorod on 27th May 1924. Already during his first class at secondary school at the age of eleven, he was fascinated by chemistry and organized a small laboratory equipped with various reagents of mystifying designations. His interest in chemistry continued at the Higher Industrial Chemical School and, after the Second World War, at the Technical University in Prague where he graduated in 1947. There he met a number of famous scientists, e.g., František Čůta, Oldřich Tomíček and Jaroslav Heyrovský, who influenced his future scientific direction. Another famous person who had a crucial impact on his career, was Professor Erica Cremer. Her first publications in the journal *Elektrochemie* on the separation of simple gases inspired him to look for a solution applicable in laboratory practice.

In 1954 he obtained the MS degree at the Technical University in Ostrava where his thesis was "Metamorphosis of deep water in sedimentary basins." In 1964 he defended the thesis "Chromatographic semimicroanalysis of gases" and was granted the title DS. He was promoted to Associate professor in analytical chemistry in 1965 at the Faculty of Natural Sciences of the University of J. E. Purkyně (as Masaryk University in Brno was known at that time). He titled his thesis "New methods for identification and determination of organic substances." He had been giving lectures on analytical separation methods there for more than two decades and in 1993 he was granted an external professorship in analytical chemistry.

Dr. Janák launched his professional career at the Chemical Works Litvínov near Most as an analytical chemist responsible for control and development of methods suitable for testing waste waters, production of phenols, synthetic methanol, formaldehyde and formic acid. He was well-versed in low pressure liquid and paper chromatography and ion exchange. It may be of interest that early in his career he reached a practical separation of hydrogen, nitrogen carbon monoxide and methane on a column filled by charcoal using carbon dioxide as a mobile phase. The newly established, geologically-oriented Institute for Petroleum Research in Brno offered Professor Janák a chance to build analytical laboratories for prospecting and evaluating sources of crude oil and natural gas shells and composition of deep ground water. It was here where he has developed the final design of the gas chromatograph and realized the analytical separation of hydrocarboneous and permanent gases. Jaroslav Janák's earliest scientific results were a breakthrough and the instrumental solution he designed led in 1952 to the first patent in the world devoted to a gas

chromatograph. He also studied geochemistry and applied ion exchange equilibria known as ion exchange chromatography to changes in the composition of deep ground waters migrating through sedimentary rocks.

Janak's gas chromatograph donated by the B.P. Research Centre, Sunbury-on-Thames, U.K. to Dr. J. Janak in 1983.

The second exemplar is in the Science Museum in London.

In 1956, the presidency of the Czechoslovak Academy of Sciences asked him to establish, chair and profile the Laboratory for Gas Analysis, later Institute of Instrumental Analytical Chemistry, now known as the Institute of Analytical Chemistry in Brno. Under his management and in the first twenty years of its existence the Institute became known world wide as the school of gas chromatography, and some of distinguished scientists are grateful to Professor Janák and the Institute for their early scientific development, e.g., M. Novotny (USA), F. I. Onuska and V. M. Bhatnagar (Canada), V. G. Berezkin (Russia), O. K. Guha, R. N. Nigan (India), J. Nuñez, L. F. Gonzáles (Cuba), N. Ruseva-Rakshieva, K. Lekova (Bulgaria), R. Staszewski (Poland).

In the first twenty years of the Institute's existence it has become well known beyond the then Czechoslovakia due to a number of world class scientific publications, patented research results and advanced instrumentation Jaroslav Janák's main scientific interests in chromatography were focused on the methodology and instrumentation of the separation of gases and trace analysis. Professor Janák with his co-workers achieved success in gas analysis, defined pyrolysis in GC conditions, multi-dimensional chromatography (GC-TLC), use of zeolits in GC, and elemental analysis. Many of his co-workers attained a high quality level of scientific excellence and opened their own projects (e.g., capillary HPLC, isotachophoresis, zone electrophoresis, supercritical fluid chromatography and extraction, etc.).

Professor Janák is author or co-author of more than 300 original papers and co-author of seven books. Analytical data per se did not interest him, but their understanding and interpretation attracted his attention. He attached importance to analytical data as a reflection of observed processes, and analytical chemistry as an

independent branch of natural science and analysis as one of the philosophical domains. During his carreer he was a visiting scientist at the Technical University in Gdańsk (Poland), New York Academy of Sciences (New York, USA), Academia Sinica in Dalian (China), Laboratorio di Cromatographia CNR, Rome (Italy) and the Technical University in Delft (The Netherlands).

Jaroslav Janák was deeply involved in establishing reputable international journals such as, *Journal of Chromatography* and *Journal of Gas Chromatography*, now *Journal of Chromatographic Science.* He had a considerable influence on the scientific profiling of the first one, and served as an editorial board member or editor of several special issues and bibliographic service for many decades. He was advisor and co-author of the compendium *Encyclopedia of Separation Science* (London 1996, UK).

Professor Janák´s contributions to the development of (analytical) chemistry, especially chromatography, have been recognized and honored with several awards and medals, namely, the M.S. Tswett Award for Distinguished Research in Chromatography (Munich, Germany 1975), the J. Heyrovsky Gold Medal (Prague, Czechoslovakia 1984), gold medal of University in Ferrara (Italy 1991), gold medal of Masaryk University in Brno (Czech Republic, 1991), medal of Faculty of Chemistry, Technical University in Brno (1992), gold medal, and "Leading Intellectual of the World" award of American Biographical Society (Raleigh, NC USA, 2004) and „De Scientia et Humanitate Optime Meritis" by the Academy of Sciences of the Czech Republic in 2005.

Since 1945, Professor Janák has been a member of the Czech Chemical Society and a long-term chairman of its office in Brno. He organized postgraduate and summer courses and due to his efforts the Chemical Faculty of Technical University in Brno was renewed. In recognition of his work he was awarded *doctor honoris causa*. This curriculum vitae would not be complete without mentioning Jaroslav's strong cultural background and his interests in everything taking place in the culture, politics, and society. For all his work and contributions to the city he was awarded the Prize of the City of Brno in 2009.

Program - CECE 2014

Monday, October 20

8:00 – 16:00 Registration

9:00 – 9:10 CECE Junior opening

9:10 – 9:25 **LA-ICP-MS AS A TOOL FOR ELEMENTAL MAPPING**

Tereza Warchilová[1,2], Tomáš Vaculovič[1,2], Zuzana Čadková[3], Vítězslav Otruba[1], Jiřina Száková[4], Viktor Kanický[1,2]

[1]Department of Analytical Chemistry, Masaryk University, Brno, Czech Republic; [2]Central European Institute of Technology, Masaryk University, Brno, Czech Republic; [3]Department of Zoology and Fisheries, Czech University of Life Sciences, Praha, Czech Republic; [4]Department of Agroenvironmental Chemistry and Plant Nutrition, Czech University of Life Sciences, Praha, Czech Republic

9:25 – 9:40 **THIOL-ENE-BASED MONOLITHIC MICROREACTORS**

Jakub Novotný[1,2], Josiane P. Lafleur[3], Jörg P. Kutter[3]

[1]Institute of Analytical Chemistry of the ASCR, v. v. i., Brno, Czech Republic; [2]Department of Biological and Biochemical Sciences, Faculty of Chemical Technology, University of Pardubice, Pardubice, Czech Republic; [3]Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

9:40 – 9:55 **POLYELECTROLYTE MULTILAYER COATINGS FOR THE SEPARATION OF PROTEINS BY CAPILLARY ELECTROPHORESIS: INFLUENCE OF POLYELECTROLYTE NATURE**

Samya Bekri, Laurent Leclercq, Hervé Cottet

Institut des Biomolécules Max Mousseron (UMR CNRS 5247), Montpellier, France

9:55 – 10:10 ***IN VITRO* RNA RELEASE OF A HUMAN RHINOVIRUS FOLLOWED VIA A MOLECULAR BEACON AND CHIP ELECTROPHORESIS**

Victor U. Weiss[1], Dieter Blaas[2], Guenter Allmaier[1]

[1]Vienna University of Technology, Institute of Chemical Technologies and Analytics, Vienna, Austria; [2]Max F. Perutz Laboratories (MFPL), Vienna Medical University, Vienna, Austria

10:10 – 10:25 **DEVELOPMENT OF A NOVEL RP-HPLC METHOD FOR THE DETERMINATION OF AMINO SUGARS IN SAMPLES OF ENVIRONMENTAL ORIGIN**

Erik Beňo, Róbert Góra, Milan Hutta

Department of Analytical Chemistry, Faculty of Natural Sciences, Comenius University in Bratislava, Bratislava, Slovak Republic

10:25 – 10:40 **HPLC DETERMINATION OF METHIONINE, HOMOCYSTEINE AND CYSTEINE ENANTIOMERS IN SERUM OF PATIENTS AFTER STROKE**

Zuzana Deáková[1,2], Zdeňka Ďuračková[2], Ingrid Žitňanová[2], Jozef Lehotay[1]

[1]Institute of Analytical Chemistry, Faculty of Chemical and Food Technology, Slovak University of Technology, Bratislava, Slovak Republic; [2]Institute of Medical Chemistry, Biochemistry and Clinical Biochemistry, Faculty of Medicine, Comenius University, Bratislava, Slovak Republic

10:40 – 11:00 Coffee break

11:00 – 11:15 **CAPILLARY ELECTROPHORETIC ANALYSIS OF SINGLE BREATH - POSSIBLE OR NOT?**

Michal Greguš[1,2], František Foret[1], Petr Kubáň[1,2]

[1]Bioanalytical Instrumentation, CEITEC MU, Brno, Czech Republic; [2]Department of Chemistry, Masaryk University, Brno, Czech Republic

11:15 – 11:30 **ANALYSIS AND CHARACTERIZATION OF ANTIMICROBIAL PEPTIDES BY CAPILLARY ELECTROPHORESIS**

Tereza Tůmová[1,2], Lenka Monincová[1], Václav Čeřovský[1], Václav Kašička[1]

[1]Institute of Organic Chemistry and Biochemistry AS CR, v.v.i., Prague, Czech Republic; [2]Institute of Chemical Technology, Prague, Czech Republic

11:30 – 11:45 **TAIL-LABELED OLIGONUCLEOTIDE PROBES FOR A DUAL ELECTROCHEMICAL MAGNETIC IMMUNOPRECIPITATION ASSAY OF DNA-PROTEIN BINDING**

Monika Hermanová, Jan Špaček, Petr Orság, Miroslav Fojta

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

11:45 – 12:00 **MAGNETIC BEAD-BASED IMMUNOCAPTURE OF CLINICAL BIOMARKERS IN MICROFLUIDIC DEVICES: FROM PEPTIDES TO WHOLE CELLS**

Zuzana Svobodova, Barbora Jankovičová, Jana Kučerová, Zuzana Bilkova

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

12:00 – 12:15 **DIODE LASER THERMAL VAPORIZATION – NOVEL SAMPLE INTRODUCTION TECHNIQUE FOR ICP MS**

Antonín Bednařík[1], Pavla Foltynová[1], Iva Tomalová[1], Viktor Kanický[1,2], Jan Preisler[1,2]

[1]Department of Chemistry, Faculty of Science, Masaryk University, Brno, Czech Republic; [2]Central European Institute of Technology (CEITEC), Masaryk University, Brno, Czech Republic

12:15 – 13:45 Lunch break - poster session

13:45 – 14:00 **A RAPID IDENTIFICATION OF TRIACYLGLICEROLS AND PHOSPHOLIPIDS USING MALDI-TOF MS**

Justyna Walczak, Bogusław Buszewski

Department of Environmental Chemistry and Bioanalysis, Faculty of Chemistry, Interdisciplinary Centre of Modern Technologies, Nicolaus Copernicus University, Torun, Poland

14:00 – 14:15 **COMPARISON OF CHIRAL STATIONARY PHASES BASED ON CYCLOFRUCTAN IN NORMAL PHASE LIQUID CHROMATOGRAPHY**

Marianna Moskaľová, Taťána Gondová

Department of Analytical Chemistry, Faculty of Science, P. J. Šafárik University, Košice, Slovak Republic

14:15 – 14:30 **SIMULATION OF MICROFLUIDIC SYSTEMS WITH COMSOL MULTIPHYSICS**

Andrea Nagy[1], Eszter Tóth[2], Kristóf Iván[2], Attila Gáspár[1]

[1]Department of Inorganic and Analytical Chemistry, University of Debrecen, Debrecen, Hungary; [2]Faculty of Information Technology and Bionics, Pázmány Péter Catholic University, Budapest, Hungary

14:30 – 14:45 **INFLUENCE OF MASS SPECTROMETRY RESOLUTION ON METABOLITE COVERAGE IN PLASMA**

Lukas Najdekr[1,2], David Friedecky[1], Ralf Tautenhahn[3], Junhua Wang[3], Tomas Pluskal[4], Yingying Huang[3], Tomas Adam[1,2]

[1]Laboratory of Metabolomics, Institute of Molecular and Translational Medicine, University Hospital and Palacky University in Olomouc, Olomouc, Czech Republic; [2]Department of Clinical Biochemistry, University Hospital in Olomouc, Olomouc, Czech Republic; [3]Thermo Fisher Scientific, San Jose, CA, USA; [4]Okinawa Institute of Science and Technology, Okinawa, Japan

14:45 – 15:00 **STUDY ON SILVER IMMOBILIZATION TO LACTOFERRIN**

Paweł Pomastowski, Bogusław Buszewski

Department of Environmental Chemistry and Bioanalytics, Faculty of Chemistry, Interdisciplinary Centre for Modern Technologies, Nicolaus Copernicus University, Toruń, Poland

15:00 – 15:20 Coffee break

15:20 – 15:35 **DEVELOPMENT AND APPLICATIONS OF IONIZATION TECHNIQUES IN AMBIENT MASS SPECTROMETRY**

Jan Rejšek[1,2], Vladimír Vrkoslav[1], Josef Cvačka[1]

[1]Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, v.v.i., Prague, Czech Republic; [2]Department of Analytical Chemistry, Faculty of Science, Charles University in Prague, Prague, Czech Republic

15:35 – 15:50 **COMPARISON OF ANTIOXIDANT PROPERTIES OF DIFFERENT MENTHA PIPERITA SPECIES AND COMMERCIAL TEAS BY CAPILLARY ZONE ELECTROPHORESIS AND SPECTROSCOPY**

Vendula Roblová[1], Miroslava Bittová[1], Petr Kubáň[1,3], Vlastimil Kubáň[1,2]

[1]Department of Chemistry, Faculty of Science, Masaryk University, Brno, Czech Republic; [2]Department of Food Technology, Faculty of Technology, Tomas Bata University, Zlín, Czech Republic; [3]Bioanalytical Instrumentation, CEITEC MU, Brno, Czech Republic

15:50 – 16:05 **TOWARDS CYTOCHROME P450 IMER FOR KINETICS AND INHIBITION STUDIES USING CAPILLARY ELECTROPHORESIS IN ONLINE CONFIGURATION**

Jan Schejbal, Roman Řemínek, Zdeněk Glatz

Department of Biochemistry, Faculty of Science and CEITEC, Masaryk University, Brno, Czech Republic

16:05 – 16:20 **WATER UPTAKE ON SILICA-BASED STATIONARY PHASES IN HYDROPHILIC INTERACTION CHROMATOGRAPHY**

Jan Soukup, Pavel Jandera

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

16:20 – 16:35 **GRAPE POMACE APPLICATION IN ENVIRONMENTAL STUDIES: FROM WASTE TO NATURAL FOOD PRESERVATIVE AND SOURCE OF BIOFUEL**

Zorana Andonovic[1], Violeta Ivanova Petropulos[2]

[1]Secondary School Yahya Kemal College, Skopje, Republic of Macedonia; [2]Faculty of Agriculture, University "Goce Delčev", Štip, Republic of Macedonia

16:35 – 17:05 **MULTILEVEL CHARACTERIZATION OF ANTIBODY THERAPEUTICS BY CESI-MS**

András Guttman

University of Pannonia, Veszprem, Hungary; University of Debrecen, Hungary; Sciex Separations, Brea, CA, USA

17:05 – 09:00 Poster session

Tuesday, October 21

8:00 – 15:00 Registration

9:00 – 9:15 CECE 2014 - Opening remarks

9:15 – 9:45 **NEXT-GENERATION PROFILING OF HUMAN IMMUNE REPERTOIRES**

Jan Berka
Roche Molecular Systems, Pleasanton, USA

9:45 - 10:15 **THE EVOLUTION OF FORM AND FUNCTION SANS GENES**

Keith Baverstock
Department of Environmental Science, University of Eastern Finland, Kuopio, Finland

10:15 – 10:45 Coffee break

10:45 – 11:15 **ANALYSIS OF BIONANOPARTICLES BY MEANS OF NANO ES/CHARGE REDUCTION COUPLED TO DIFFERENTIAL MOBILITY ANALYZER**

Guenter Allmaier[1], Victor Weiss[1], Marlene Havlik[1], Martina Marchetti-Deschmann[1], Peter Kallinger[2], Wladyslaw Szymanski[2]
[1]Institute of Chemical Technologies and Analytics, Vienna University of Technology (TU Wien), Vienna, Austria; [2]Faculty of Physics, University of Vienna, Vienna, Austria

11:15 - 11:45 **THE STUDY OF ULTRASMALL SAMPLES BY FAST CAPILLARY ELECTROPHORESIS - MASS SPECTROMETRY**

Frank-Michael Matysik, Jonas Mark, Marco Grundmann, Sven Kochmann, Andrea Beutner
University of Regensburg, Institute for Analytical Chemistry, Chemo- and Biosensors, Regensburg, Germany

11:45 - 12:15 **TIME-RESOLVED CRYO-ELECTRON MICROSCOPY OF MACROMOLECULES**

Tanvir Shaikh
CEITEC, Brno, Czech Republic

12:15 – 14:15 Lunch break – poster session

14:15 – 14:45 **IMPROVING ENANTIOSELECTIVITY AND RESOLUTION IN CEC AND NANO-LC: RECENT RESULTS**

Salvatore Fanali

Institute of Chemical Methodologies, Italian National Research Council (C.N.R.), Monterotondo, Italy

14:45 – 15:15 **THE VALUE OF BIOBANK PATIENT SAMPLES IN PROTEIN EXPRESSION STUDIES**

György Marko-Varga

[1]Clinical Protein Science & Imaging Group, BioMedical Center, University of Lund, Lund, Sweden; [2]Dept. of Surgery, Tokyo Medical University, Tokyo, Japan

15:15 – 15:45 **SEX DURING COMMUNISM. INTIMATE LIFE AND THE POWER OF EXPERTISE**

Kateřina Lišková

Masaryk University, Brno, Czech Republic

16:10 City walk with invited speakers

19:00 Conference dinner with the traditional Moravian music

Wednesday, October 22

9:15 – 10:00 **THE MONKEY KING AND PIGSY FERRYING THE PROTEOMIC SUTRAS INTO THE THIRD MILLENNIUM**
Pier Giorgio Righetti
Department of Chemistry, Materials and Chemical Engineering "Giulio Natta", Politecnico di Milano, Milano, Italy

10:00 – 10:30 **HYDROPHILIC INTERACTION CHROMATOGRAPHY- THE STATE OF THE ART**
David McCalley
Faculty of Health and Life Sciences, University of the West of England, Bristol, UK

10:30 – 11:00 Coffee break

11:00 – 11:30 **MICROFLUIDICS COUPLED WITH MASS SPECTROMETRY FOR ONLINE MONITORING OF DYNAMIC BIOLOGICAL PROCESSES**
Ryan T. Kelly
Pacific Northwest National Laboratory, Richland, USA

11:30 - 12:00 **HARSH ENVIRONMENT CAPILLARY ELECTROPHORESIS**
Mihkel Kaljurand
Tallin University of Technology, Tallinn, Estonia

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

14:00 – 14:30 **EVOLUTION OF A MICROFLUIDIC LC/MS SYSTEM - FUNDAMENTAL TECHNOLOGY TO A COMPLETED SYSTEM**
Geoff Gerhardt
Waters Corporation, Milford, MA, USA

14:30 – 15:00 **A LIGHT AT THE END OF THE TUNNEL FOR THE COMPREHENSIVE COMPOUND IDENTIFICATION IN UNTARGETED METABOLOMICS**
Robert Mistrik
HighChem Ltd., Bratislava, Slovak Republic

15:00 – 15:30 **cHiPLC: MICROFLUIDIC COMPONENTS FOR HPLC**
Don W. Arnold
Eksigent, Redwood City, CA, USA

15:30 - 16:00 **SUB-2 µM SILICA PARTICLES WITH INTERNAL MACROPORES: DO WE NEED ANOTHER PARTICLE TYPE IN CAPILLARY SEPARATIONS?**
Milos V. Novotny[1], Sara E. Skrabalak[1], James P. Grinias[2], Justin M. Godinho[2], James W. Jorgenson[2]
[1]Department of Chemistry, Indiana University, Bloomington, USA; [2]Department of Chemistry, Kenan Laboratory, University of North Carolina, Chapel Hill, USA

16:00 Closing remarks

List of poster presentations

P1 ANALYSIS OF SEQUENCE SPECIFIC INTERACTIONS BETWEEN DNA AND P53 FAMILY PROTEINS BY ELISA, SLOT-BLOT AND EMSA
Matej Adámik, Lucie Holaňová, Lucie Navrátilová, Jana Nygrínová, Jana Pokorová, Marek Petr, Vlastimil Tichý, Marie Brázdová

P2 SAMPLE PREPARATION FOR SINGLE CELL ANALYSIS
Eva Adamová, Evgenia Yu. Basova, Anna Potáčová, František Foret, Eva Matalová, Karel Klepárník

P3 LAYER-BY-LAYER CAPILLARY COATING FOR MODIFICATION OF ELECTROOSMOTIC FLOW IN CAPILLARY ELECTROPHORESIS
Daniel Baron, Jana Horská, Jan Petr

P4 CITP ANION ANALYSIS OF BEVERAGES
Bartošková M., Pelikánová B., Lubal P., Farková M.

P5 THE DETERGENT EFFECT DURING N-GLYCAN RELEASE FROM STANDARD GLYCOPROTEINS
Judit Bodnar, Andras Guttman

P6 SIMULTANEOUS ANALYSIS OF DEXRAZOXANE AND ITS PUTATIVE ACTIVE METABOLITE USING HPLC-MS/MS
Jan Bures, Vit Sestak, Hana Jansova, Marek Kratochvil, Jaroslav Roh, Jiri Klimes, Petra Kovarikova

P7 SPECTROPHOTOMETRIC DETERMINATION OF METALLO-THIONEINS IN FISH
Bušová M., Havlíková G., Opatřilová R.

P8 MONITORING OF SELECTED PARAMETERS IN AMELANCHIER ALNIFOLIA EXTRACTS BY UV-VIS-NIR AND EPR SPECTROSCOPY
Butorová Lenka, Polovka Martin Vítová Eva

P9 EFFECT OF SLEEVE GASTRECTOMY ON PARAMETERS OF BONE METABOLISM AFTER RADICAL WEIGHT LOSS
Bužga Marek, Švagera Zdeněk

P10 BEAD-BASED IMMUNOSENSOR FOR EARLY STAGE DIAGNOSIS OF OVARIAN CANCER
Michaela Čadková, Veronika Dvořáková, Radovan Metelka, Zuzana Bílková, Lucie Korecká

P11 USE OF PCR-DGGE FOR CONTROL OF BACTERIA IN CHEESES AND THEIR PICKLES
Čakajdová Martina, Trachtová Štěpánka, Mohelský Tomáš, Němečková Irena, Španová Alena, Rittich Bohuslav

P12 PHOTO-INDUCED FLOW-INJECTION DETERMINATION OF NITRATE IN WATER
Pavel Mikuška, Lukáš Čapka, Zbyněk Večeřa, Ivan Kalinichenko, Josef Kellner

P13 OPTIMIZATION OF AMINO ACID DERIVATIZATION PROCEDURE USING NAPHTHALENE-2,3-DICARBOXALDEHYDE FOR CAPILLARY ELECTROPHORESIS WITH FLUORESCENCE DETECTION
Andrea Cela, Tereza Dedova, Ales Madr, Zdenek Glatz

P14 MODELLING OF MICROFLUIDIC FLOW-GATING INTERFACE FOR TWO-DIMENSIONAL LIQUID CHROMATOGRAPHY-CAPILLARY ELECTROPHORESIS
Petr Česla, Jana Křenková, Tomáš Václavek, Jana Váňová, Nikola Vaňková, Jan Fischer

P15 INFLUENCE OF PEG 6000 CONCENTRATION ON DNA VISCOSITY AND ADSORPTION CAPACITY ON MAGNETIC MICROSPHERES
Maria Chroboková, Judit Tóth, Alena Španová, Bohuslav Rittich

P16 MONOSACCHARIDE ANHYDRIDES IN ATMOSPHERIC AEROSOLS – OPTIMIZATION OF SEPARATION AND DETECTION BY MEANS OF LC-MS TECHNIQUE
Richard Čmelík, Pavel Coufalík, Pavel Mikuška

P17 EXTRACTION OF BIOAVAILABLE PROPORTION OF METALS IN DIESEL EMISSIONS AND ATMOSPHERIC AEROSOLS USING ARTIFICIAL LUNG FLUIDS
Pavel Coufalík, Pavel Mikuška, Kamil Křůmal, Zbyněk Večeřa, Michal Vojtíšek, Tomáš Matoušek

P18 TOWARDS THE PRODUCTION OF HIGHLY EFFICIENT POLYMERIC MONOLITHIC COLUMNS FOR HILIC SEPARATIONS OF SMALL MOLECULES
Sinéad Currivan, Pavel Jandera

P19 TESTING DIFFERENT TYPES LYSIS METHOD FOR THE PREPARATION OF THE BACTERIAL CELL LYSATES WITH SUBSEQUENT ISOLATION AND AMPLIFICATION OF DNA
Robert Čuta, Michaela Fričová, Bohuslav Rittich, Alena Španová

P20 QUANTUM DOT-BASED FÖRSTER RESONACE ENERGY TRANSFER BIOANALYSIS
Vladimíra Datinská, Karel Klepárník, Barbora Belšánová, Marek Minárik, František Foret

P21 MAGNETIC BEAD MEDIATED COMBINATORIAL CARBOHYDRATE SYNTHESIS
Boglárka Döncző, László Kalmár, András Guttman

P22 IDENTIFICATION OF HUMAN MICROBIOME IN HARD-TO-HEAL WOUNDS BY MALDI-TOF MS AND SEQUENCING
Dagmar Chudobova, Kristyna Cihalova, Roman Guran, Simona Dostalova, Kristyna Smerkova, Radek Vesely, Jaromir Gumulec, Zbynek Heger, Michal Masarik, Vojtech Adam, Rene Kizek

P23 NON-INVASIVE SAMPLING AND SUBSEQUENT CAPILLARY

ELECTROPHORETIC ANALYSIS OF SWEAT AND SALIVA IN CLINICAL DIAGNOSIS OF CYSTIC FIBROSIS
P. Ďurč, M. Greguš, J. Hodáková, E. Pokojová, J. Skřičková, F. Foret, P. Kubáň

P24 QUANTUM DOT-BASED LABELING PROCEDURES FOR PREPARATION OF SPECIFIC ANTIBODY CONJUGATES
Veronika Dvorakova, Michaela Cadkova, Barbora Jankovicova, Zuzana Bilkova, Lucie Korecka

P25 ANALYSIS OF SACCHARIDES IN GRAPES AND WINE USING HPLC WITH VARIOUS STATIONARY PHASES
Dana Flodrová, Richard Čmelík, Jiří Šalplachta, Janette Bobáľová

P26 THE STUDY OF INFLAMMATORY MARKERS OF ONCOLOGICAL PATIENTS BY MULTIVARIATE DATA ANALYSIS
Ľuboš Fraňo, Pavel Májek

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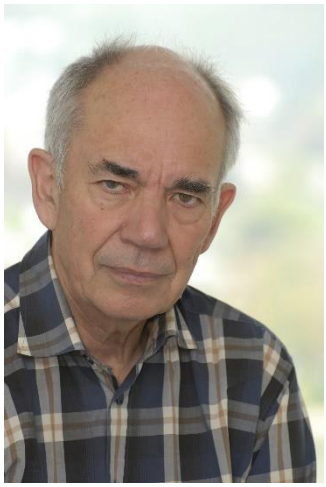
About the invited speakers

András Guttman, Professor of Translational Glycomics (Horváth Laboratory of Bioseparation Sciences, University of Pannonia and Debrecen, Hungary) also leads the application efforts at Sciex Separations (Brea, CA). His work is focusing on capillary electrophoresis and CE-MS based proteomics and glycomics analysis of biopharmaceutical and biomedical interests. Dr Guttman previously held academic appointments at Northeastern University (Boston, MA) and University of Innsbruck (Austria) as well as industrial positions at Novartis (La Jolla, CA), Genetic BioSystems (San Diego, CA), and Beckman Coulter (Fullerton, CA), developing high resolution capillary electrophoresis and microfluidics based separation methods. Professor Guttman has more than 240 scientific publications, wrote 32 book chapters, edited several textbooks and holds 19 patents. He is a CASSS board member, president of the Hungarian Chapter of the American Chemical Society, and on the editorial boards of numerous international scientific journals. Dr. Guttman graduated from University of Veszprem, Hungary in chemical engineering, where he also received his doctoral degree. He was recognized by the Analytical Chemistry Award of the Hungarian Chemical Society in 2000, elected as a member of the Hungarian Academy of Sciences in 2004, named as Fulbright Scholar in 2012, received the CASSS CE Pharm Award in 2013, the Arany Janos medal of the Hungarian Academy of Sciences in 2014 and the Pro Scientia award of the University of Pannonia just recently.

Jan Berka is a Director of Research, Sequencing Unit, Roche Molecular Systems in Pleasanton, CA, since April 1, 2014. Jan was born in the Czech Republic where he graduated from Masaryk University with a Doctor of Natural Sciences degree (RNDr.) in molecular biology and genetics. After four years of post-graduate studies, Jan received a post-doctoral fellowship at the Barnett Institute, Northeastern University in Boston, MA, where he worked on developing multi-capillary DNA sequencers, a project funded by the DOE under the Human Genome program. Jan continued to develop DNA analysis methods at CuraGen and in April 2000 became a team member at 454 Life Sciences, a CuraGen spin-off. At 454 life Sciences, he was a co-inventor of several key components of the Roche-454 DNA sequencing system, including emulsion PCR. In 2006, Jan switched focus to applying next-generation 454 sequencing to the discovery of rare genetic variants associated with human disease traits, first at Perlegen Sciences with David

Cox, and later at Pfizer Rinat Laboratories in South San Francisco. At Rinat, Jan's team was among the first groups to apply NGS to profiling of the human immune system. Jan continued to explore innovative immunosequencing approaches at Adaptive Biotechnologies in Seattle, where he developed a novel method for pairing sequences of immune receptor chains, until recently.



Keith Baverstock graduated in chemistry from London University. His Ph D, also from London University, was in chemical kinetics. He was an NRC of Canada postdoctoral fellow at Atomic Energy of Canada in Pinawa, Manitoba, for 2 years, returning to the UK and Nottingham University for further postdoctoral work. In 1971 he joined the UK Medical Research Council's Radiobiology Unit with the dual remit of research and advising on public health aspects of radiation exposure. In 1991 he joined the World Health Organisation and was instrumental in uncovering the "epidemic" of childhood thyroid cancer resulting from the Chernobyl accident. On retirement from the WHO in 2003 he continued his research at the University of Kuopio, now the University of Eastern Finland. He has taken a keen interest in the public health consequences of nuclear accidents and his WHO programme was instrumental in uncovering the childhood thyroid cancer outbreak after the Chernobyl accident. Currently he collaborates with a Citizen Scientist organisation in Japan in connection with the Fukushima accident. Earlier he worked extensively on public health issues in the Marshall Islands. His recent research has concentrated on uncovering the processes that underlie the phenomenon of genomic instability and how biology has to change in order to accommodate the phenomenon. He believes the Modern Synthesis that dominates genetics is not just flawed but fundamentally wrong and needs to be replaced by a "theory of life" based on thermodynamic considerations.

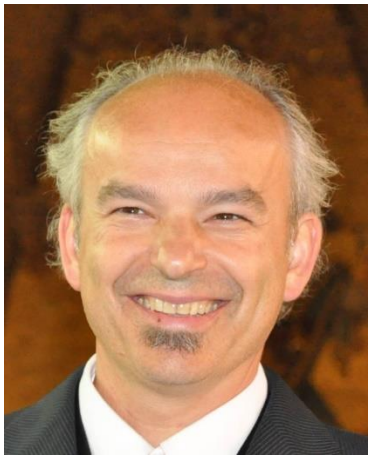
Guenter Allmaier was born and raised in Vienna (Austria), got a M. Sc. degree in Pharmaceutical Chemistry and Pharmacology from the University of Vienna and 1983 his Ph.D. degree in Analytical Chemistry from the same institution. From 1985 to 1986 he worked as post-doctoral associate in the Department of Chemistry (with Prof. K. Biemann) at MIT (Cambridge, MA, USA). Afterwards he joined the Institute of Analytical Chemistry (University of Vienna) as assistant professor. He worked several periods as visiting scientist in the Department of Molecular Biology (with Prof. P. Roepstorff) at University of Southern Denmark (Odense, Denmark) as well as visiting professor in the Centro de Biologia Molecular Severo Ochoa, C.S.I.C., Universidad de Autonoma de Madrid (Madrid Spain). 1995 he was appointed to the rank of associate professor. 2003 he moved as

full professor of analytical chemistry to the Institute of Chemical Technologies and Analytics at the Vienna University of Technology (TU Wien) and is directing the institute as managing director since 2011. He has written or co-authored more than 250 publications in peer-reviewed international journals and books as well as has been granted two patents. He has participated in the organization of several international conferences (e.g. 20th IMSC, Geneva, Switzerland; International Symposium of Glycoconjugates XXI, Vienna, Austria; 27th IMMS (Informal Meeting on Mass Spectrometry) Retz, Austria). Furthermore he was founder of the Austrian Proteomics Association (AuPA) and its annual meeting as well as the Central and Eastern European Proteomics Symposium (CEEPS) series. He acts at the moment as elected vice president of the Austrian Society of Analytical Chemistry (ASAC). He obtained several prizes as the John Beynon RCM Award and Dr. Wolfgang Houska Prize. His interests are covering mass spectrometry and separation sciences in terms of instrumentation with a broad array of application fields from nanoparticles to lipidomics and proteomics as well as tribology and biopharmaceuticals.

Frank-Michael Matysik is Professor of Analytical Chemistry at the University of Regensburg (Bavaria, Germany). He studied chemistry at the University of Leipzig and received his Ph.D. (1994) and "Habilitation" (2001) degrees from the University of Leipzig. From 2001 to 2008 he was "Privatdozent" for Analytical Chemistry at the same university. In May 2008 he accepted the position of a professor of chemistry at the University of Regensburg where he is representing the field of instrumental analytical methods. Research interests: Instrumental analytical developments, Hyphenated analytical systems, Miniaturized sample preparation techniques, Electroanalysis, Bioelectroanalysis, Electromigrative separation systems (capillary and chip format), Chromatographic separation techniques, Mass spectrometry.

Tanvir (Tapu) Shaikh is a junior group leader at the Central European Institute of Technology (CEITEC). Tapu received his Ph.D. in the laboratory of David DeRosier, and spent his postdoctoral years at the Wadsworth Center in Albany, working under Joachim Frank, Rajendra Agrawal, Terry Wagenknecht, and ArDean Leith. Tapu's research interests are two-fold: development of methodologies for time-resolved electron microscopy (EM) and development of the SPIDER image-processing suite. The time-resolved methodologies have included flash photolysis and, mostly recently, development of microfluidic devices which quickly mix two solutions and spray microdroplets onto an EM grid. The SPIDER software suite came into existence in 1978 and is used.

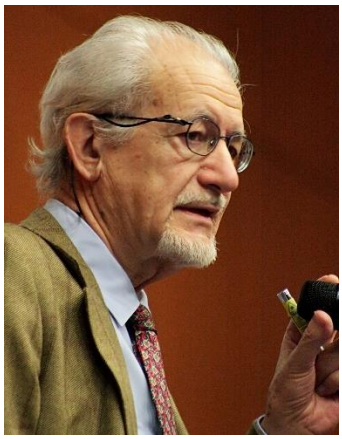
Salvatore Fanali is a Senior Researcher "Direttore di Ricerca" at the Italian National Research Council (C.N.R.), Institute of Chemical Methodologies in Monterotondo (Rome) Italy and head of "Capillary Electromigration and Chromatographic Methods" Unit. In 1974 he received the degree of Dr. in Chemistry at Rome University "La Sapienza" and later on the PhD in Analytical Chemistry at Comenius University – Bratislava, Slovakia. His research is focused on development of miniaturized techniques, e.g., nano-liquid chromatography/nano-LC, capillary zone electrophoresis/CZE, capillary electrochromatography /CEC. They were coupled with mass spectrometry. Studies on enantiomers separations, new stationary phases are carried out. Methods are applied to pharmaceutical, agrochemical, food, environmental, forensic analysis. He is author or co-author of about 300 publications in Journal (SCI) of international interest, chapters in books, two booklets. He received awards, e.g., Bratislava University, University of Verona, "Liberti" Medal in Analytical Chemistry (Italian Chemical Society), "Nota" Medal (ISCC-2014) Capillary Chromatography, University of Olomouc. He is Editor of Journal of Chromatography A (Elsevier), honorary Editor in Journal of Separation Science where he served as Editor-in-chief and member of the advisory editorial board of 6 International Journals.



Gyorgy Marko-Varga (GMV) is Professor at the Tokyo Medical University, Japan, and is the head of the div. Clinical Protein Science and Imaging. GMV has been working within senior Drug-, Discovery/Development positions and responsibilities within Astra, and AstraZeneca for a period of more than 20 years. He started as a Lead Scientist in collaboration with the Nobel Prize winner Bengt Samuelsson, Karolinska Institute, on inflammation studies in 1992. GMV has been in leadership positions in AstraZeneca; as global proteomics head, Clinical Biomarker Platforms used in clinical studies phase I and II, moving into phase III, and Biological Mass Spectrometry. In 2006 GMV was one of the initiators of "Nietorp AB", a MicroTechnology company within AstraZeneca. In addition Marko-Varga has been a founder of additionally two start-up companies, ISET AB (2006) and OKRAM Technology (2009). He was responsible for IRESSA Protein Biomarker Discovery studies in Japan (2005-2009) with 52 Lung Cancer Clinical Centres throughout Japan, the biggest Biomarker study activities in the industry with 4.000 patients. Today GMV is Responsible for Biobank and Biomarker developments within the "Big 3" study: Lung Cancer-Cardiovascular diseases-, and COPD with 100,000 patients processing 5 Million samples (2012-2015) in Southern Sweden. He became the

leading PI of a 5-year project in the Malignant Melanoma, sponsored by the Kamprad Foundation (2012-2017), of a 5-year grant on Protein Biomarker Discovery and Drug Imaging for Cancer Research. Marko-Varga has since then been the PI or Co-PI of several national and international grants, including one funded from the Swedish Strategic Foundation funded in 2011 on Cardiac Infarct. Additional Biobanking studies within Lung Cancer and COPD runs under the leadership of Marko-Varga. Marko-Varga has published more than 270 scientific publications since 1984 in reviewed international scientific journals, 21 Book Chapters and Edited 2 Books, His H index for the global career amounts to more than 7000 citations and H-index of 42 (2014-02-01). As part of his career, he is the founder and President of the Swedish Proteomics Society, General Council member (Swedish representative). I became the President of the European Proteomics Association (EuPA) 2011-2015. Marko-Varga is also the European Editor of Journal of Proteome Research, an American Chemical Society journal. In addition Editorial board member of additionally 9 international journals. As a longstanding member (20 years) of the Swedish Academy of Pharmaceutical Sciences (Drug Analysis Section), organised and lead more than 25 national and international congresses, as well as coerces and workshops. He had 14 PhD students, 6 Licenciate students, more than 25 diploma students, 8 post docs, throughout a 19 year period. He has filed 20 patent applications in Europe as well as worldwide, and is the owner of 10 approved patents. The extensive educational role through supervision of students of GMV has been complemented by teaching coerces, and developing new pedagogic lecturing, that is combined with experimental sections. Students are experimentally introduced to cutting edge research technologies in an environment of front line science at the new medical mass spectrometry laboratories at the Biomedical Center in Lund.

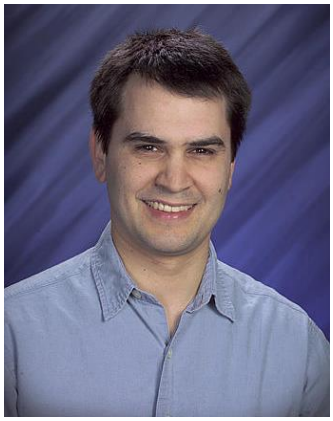
Kateřina Lišková, PhD is Assistant Professor in gender studies and sociology at Masaryk University. In the 2012/13 academic year, she was a Visiting Scholar at Columbia University where she conducted her research on sexology and sexuality in communist Czechoslovakia, supported by the Marie Curie International Outgoing Fellowship - European Commission Seventh Framework Programme. Her research is focused on gender, sexuality, and the social organization of intimacy, particularly in Central and Eastern Europe. In the past, she was affiliated with the New School for Social Research as a Fulbright Scholar and as a Visiting Scholar with New York University. She has lectured at various U.S. universities and her papers have appeared in several monographs published by Routledge, SAGE, Blackwell and Palgrave. In the Czech Republic, her book Good Girls Look the Other Way, Feminism and Pornography was published by Sociological Publishing House (2009).



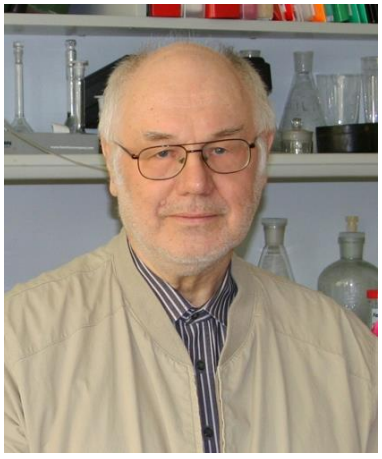
Pier-Giorgio Righetti earned his Ph. D. in Organic Chemistry from the University of Pavia in 1965. He then spent 3 years as a Post. Doc. at MIT and 1 year at Harvard (Cambridge, Mass, USA). He is full professor of Proteomics at the Milan's Polytechnic. He is in the Editorial Board of Electrophoresis, J. Proteomics, BioTechniques, Proteomics, Proteomics Clinical Applications. He has co-authored the book Boschetti, E. Righetti, P.G. *Low-Abundance Proteome Discovery; State of the Art and Protocols*, Elsevier, Amsterdam, 2013, pp. 1-341. He has developed isoelectric focusing in immobilized pH gradients, multicompartment electrolyzers with isoelectric membranes, membrane-trapped enzyme reactors, temperature-programmed capillary electrophoresis and combinatorial peptide ligand libraries for detection of the low-abundance proteome. On 560 articles reviewed by the ISI Web of Knowledge (Thomson Reuters), Righetti scores 19.500 citations, with an average of 33 citations/article and with a H-index of 61. Only in the last nine years (2005-2013) he has received citations ranging from 1000 to 1200 per year. He has won the CaSSS (California Separation Science Society) award (October 2006), at that time in its 12th edition, and the Csaba Horvath Medal award, presented on April 15, 2008 by the Connecticut Separation Science Council (Yale University). In 2011, he has been nominated honorary member of the Spanish proteomics society and in 2012 he has won the prestigious Beckman award and medal granted in February at the Geneva MSB meeting. In October 2014, in Madrid, he will be awarded the much coveted HUPO Distinguished Achievement in Proteomic Sciences and in November 2014, in Atlanta, the Prize of the American Electrophoresis Society.

David McCalley is Professor of Bioanalytical Science at the University of the West of England, Bristol U.K. In 2013, he was named as one of the world's 100 most influential analytical scientists by 'Analytical Science' magazine after a poll of its 60,000+ readers, and the input of an expert jury. He was awarded the Silver Jubilee medal of the Chromatographic Society in 2008. He serves on the Editorial Board of the Journal of Chromatography A and the magazine LC.GC, and has been a member of the Scientific Committee of a number of conferences including the HPLC 2013 symposium held in Amsterdam. In the past two years, he has given invited lectures in New Orleans, Stockholm, Utrecht, Balaton, Amsterdam, Umea, Paris, Tarragona and Anaheim. Professor McCalley's research is directed towards the understanding of the fundamental mechanisms of separation that occur in liquid and gas chromatography. These studies in LC have included the effects of pressure on the separation, superficially porous packings, overloading effects, and hydrophilic interaction chromatography. His work has also been directed towards

application of these techniques, for example in the development of robust methods for the analysis of strongly basic pharmaceuticals using LC, and the determination of sterols that are indicative of environmental pollution by GC. His work has been funded by the U.K. Engineering and Physical Science Research Council, by instrument manufacturers through donation of sophisticated analytical equipment and by the pharmaceutical industry.



Ryan T. Kelly is a Senior Research Scientist and leads the Instrument Development and Microfabrication laboratories at the William R. Wiley Environmental Molecular Sciences Laboratory (EMSL), a national scientific user facility at Pacific Northwest National Laboratory in Richland, Washington, USA. He has a broad background in the design and fabrication of microfluidic devices and other microsystems for a diverse array of applications. His graduate research at Brigham Young University (Ph.D. 2005) focused on the development of novel electrically driven separation and analysis methods for proteins and peptides within microfluidic devices. While at PNNL, he developed approaches for increasing the sensitivity of electrospray ionization mass spectrometry using both capillary-based and microfabricated systems as well as improved ion optics. His process for chemically etching electrospray emitters for dramatically improved performance in the nanoflow regime has recently been commercialized and the multi-nanoelectrospray sources that he developed were a key component of the R&D 100 award-winning, "Ultrasensitive electrospray ionization source and interface". His current research interests involve coupling microfluidic systems with mass spectrometry for sample-limited bioanalyses and to provide solution based, label-free determination of kinetic parameters for biomedical and bioenergy research. Kelly has authored or co-authored more than 50 scientific publications and is an inventor on eight issued and three pending patents.



Mihkel Kaljurand is professor and director of the Institute of Chemistry which is based in the Tallinn University of Technology, Tallinn, Estonia. Prior to joining to Tallinn University of Technology, Prof. Kaljurand served as the senior researcher at Estonian Academy of Sciences, where he also received his candidate of chemistry degree in 1979 (Leningrad University) and doctor of sciences degree (Moscow Institute of Physical Chemistry) in 1991. He has worked with a number of public organizations in his research endeavors, including NASA and Southern Illinois University (Carbondale IL, USA). Mihkel Kaljurand has been awarded Estonian Science Prize (twice) and he has been awarded fellowships by the Fulbright Visiting Scholar Program (y. 2002) and the National Research Council Research

Associateship Program (y. 1995-1996). His teaching interests and experience are in the areas of analytical chemistry. He has written extensively on chemometrics, instrumental analysis and separation science. Dr. Kaljurand's current research focuses on automatization and miniaturization of capillary electrophoresis and on green analytical chemistry.

Geoff Gerhardt got his start in analytical chemistry as Chief Bottle Washer at a water analysis lab. While cleaning sample collection bottles was his primary responsibility there, it was his understanding and maintenance of a rather antiquated LIMS that turned that summer internship into a full-time position. When a position came up at the Canadian Food Inspection Agency (CFIA) where more sophisticated instrumentation like LC, GC and MS were used, Geoff leapt at it. Geoff was a chemist at CFIA, developing methods for the detection of drug residues in animal tissue. While at CFIA, he completed his Ph.D. at the University of Saskatchewan with a focus on electrochemical detection for capillary electrophoresis. In the process of this research he developed a compact, automated CE-ECD system that caught the eye of the folks at J&W Scientific who were looking to develop a CE system. Geoff joined J&W Scientific in 1999 to further develop this CE system, but they were acquired by Agilent in 2000. Agilent was primarily interested in J&W's GC consumables business and had their own CE system, so Geoff moved on to Waters. Geoff has been doing instrument development at Waters for 14 years and now leads the Core Research Group which has a charter to identify, develop and prototype next-generation analytical solutions. With expertise in the three major areas of Waters' technology: Separations science, mass spectrometry and informatics, Core Research looks for innovative technologies that provide integration opportunities.

Robert Mistrik received a master degree from the Slovak Technical University, Bratislava, Slovakia in 1991 and Ph.D. from the University of Vienna, Austria in 1994. Between 1995 - 1997 he held a postdoctoral position at National Institute of Standards and Technology, Gaithersburg, MD, USA working in Mass spectrometry data centre. Back in Slovakia in 1998, he founded HighChem, Ltd., a privately owned scientific software company, and since then he is holding the position of CEO. In 2009 he has been awarded the Head of the Year price, a national award for exceptional achievements in science and technology. Dr. Mistrik was a member of scientific steering committee in METAcancer consortium aiming to identify small molecule biomarkers in breast

cancer tissue. In 20012 he has been elected into Board of Directors of the Metabolomics Society.

Don W. Arnold

Milos V. 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. Milos V. Novotny 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.

Abstracts of oral presentations – Invited speakers

MULTILEVEL CHARACTERIZATION OF ANTIBODY THERAPEUTICS BY CESI-MS

András Guttman[1,2,3]

[1]*MTA-PE Translational Glycomics Group, University of Pannonia, Veszprem, Hungary*
[2]*Horváth Laboratory of Bioseparation Sciences, University of Debrecen, Hungary*
[3]*Sciex Separations, Brea, CA, USA*

Summary
The increase of the number of approved therapeutic proteins in the market triggered rapid development of comprehensive and reproducible multilevel characterization methods for the biopharmaceutical industry and regulatory agencies. One of the largest groups of biotherapeutics is monoclonal antibodies (mABs), possessing various post-translational modifications (PTMs) and potential degradation hotspots, which should be analyzed during clone selection, manufacturing and lot release as potentially affecting efficacy and immunogenicity. The exceptional separation power of capillary electrophoresis (CE) in conjunction with high resolution mass spectrometry fulfills the multilevel characterization requirements of: Level-1) determination of accurate molecular mass and some degree of heterogeneity at the intact protein level; Level-2) measurement of exact molecular mass of the heavy and light chains as well as the degree of heterogeneity after reduction of the disulfide bonds with or without alkylation; Level 3) characterization of degradative hotspots such as aspargine-deamidation, methionine-oxidation, glutamic-acid-cyclization, C-terminal lysine heterogeneity and other posttranslational modifications at the peptide/glycopeptide level after proteolytic digestion of the reduced and alkylated antibody; Level 4) glycosylation characterization. In this presentation a comprehensive multilevel characterization example will be given for a representative monoclonal antibody illustrating the benefits of the integration of capillary electrophoresis (CE) and electrospray ionization (ESI) in a unified bioanalytical process (CESI) coupled with high resolution mass spectrometry. The low flow rate of the system (>20 nL/min) ensured maximized ionization efficiency and dramatically reduced ion suppression.

NEXT-GENERATION PROFILING OF HUMAN IMMUNE REPERTOIRES

Jan Berka

Roche Molecular Systems, Pleasanton, USA

Summary

Advances in high-throughput DNA sequencing have enabled the development of a powerful new technology for probing the adaptive immune system. Due to the vast diversity of immunoglobulin and T-cell receptor genes, deep sequencing of these loci pose a genome-size sequencing challenge. Millions of B or T cell receptor sequences can be read in parallel from a single sample, but the true size of the human immunome has not yet been experimentally determined. However, the dynamics of an adaptive immune response, which is based on clonal expansion and contraction, can be monitored in real time at high sensitivity and resolution. A number of clinical applications for this technology are presently under study. We will present examples of immune repertoire sequencing applications in therapeutic monoclonal antibody development, in autoimmune disease and hematological malignancies. Latest developments in methods for high-throughput sequencing cognate T-cell receptor chains from tens of thousands of cells will be reported.

THE EVOLUTION OF FORM AND FUNCTION SANS GENES

Keith Baverstock

Department of Environmental Science, University of Eastern Finland, Kuopio, Finland

Summary

The Modern Synthesis or Neo-Darwinism sees the development of form and function in organisms as being due to small modifications (mutations) to the genomic DNA sequence, creating genetic variation upon which natural selection acts. It was worrying to Darwin that in fact the evidence, both extant and extinct in the fossil record, does not support this gradual process in so far as there seems to be no clear case of a completely smooth transition between related species. This hypothesis underpinning genetics has up to 2001, with the completion of the sequencing of the human genome, not been directly testable. The thirteen years post the genomic sequence have not relieved Darwin's anxiety in so far as attempts to relate phenotypic traits to genomic variation have failed for common diseases and mental conditions such as schizophrenia. It is now necessary to question the role genes in biology and consequently the integrity of genetics.

Based on a reappraisal of the foundations of biology [1] it has been proposed that natural selection acts upon the efficiency with which organisms can extract energy (nutrient) from their ecosystems and that the life process is governed by the 2nd law of thermodynamics and the principle of least action proposed by Maupertuis 100 years prior to the publication of the "*The Origin*". Following the logic of a metabolism-first origin of life, preceded by proto-life based on proteins and subsequent regulation of the cell by information bearing proteins contributing to a quasi-stable attractor state representing phenotype, it can be concluded that for multi-celled organisms form arises from a cellular attractor state based on information contained in the cellular phenotype and function from the deployment of specialised cells, for example, to photosensitive cells. Genes figure only as a data base for peptide sequences which are manipulated through downward causation by the phenotype.

Reference

[1] Annila, A. and K. Baverstock, (2014) Genes without prominence: a reappraisal of the foundations of biology. J R Soc Interface. 11:20131017.

ANALYSIS OF BIONANOPARTICLES BY MEANS OF NANO ES/CHARGE REDUCTION COUPLED TO DIFFERENTIAL MOBILITY ANALYZER

Guenter Allmaier[1], Victor Weiss[1], Marlene Havlik[1], Martina Marchetti-Deschmann[1], Peter Kallinger[2], Wladyslaw Szymanski[2]

[1]Institute of Chemical Technologies and Analytics, Vienna University of Technology (TU Wien), Vienna, Austria
[2]Faculty of Physics, University of Vienna, Vienna, Austria
guenter.allmaier@tuwien.ac.at

Summary

Nano ES with charge reduction (by Po-210, corona discharge or soft X-ray irradiation) is used to form single-charge bionanoparticles (intact virus, vaccine particles, polysaccharides, liposomes or VLP-antibody complexes), followed by size separation with a differential mobility analyzer (from 2.5 up to 800 nm depending on the device) and chemical nature-independent detection as well as sample collection of size-selected bionanoparticles for further investigations. A correlation between the measured electric mobility diameter and molecular mass could be obtained finally.

1 Introduction

For the physico-chemical characterization of bionanoparticles as virus-like particles (VLPs), VLP-antibodies complex, vaccines, polysaccharides, liposomes and recombinant antibodies, besides functional parameters usually methods as the imaging techniques SEM or AFM in different modes as well as far it is feasible ESI MS and as

the separation techniques SEC, analytical ultracentrifugation, AF4 as well as MALS or DLS are applied showing pros and cons. Here, we want to present a technique, based on nano electrospray ionization (nano ES) with charge reduction to singly charged species combined with an analyzer (separation device) operated at atmospheric pressure, which is mainly targeted to analyzed bionanoparticles or –nano-objects with molecular masses beyond 100 kDa or sizes above 5 nm.

2 Experimental

The nano ES unit is integrated with a charge reduction chamber (incorporating an α-radiation source, a corona charger or a soft X-ray tube generating a bi/monopolar atmosphere) and coupled to a differential mobility analyzer (DMA; an ion mobility-based separation device) connected to condensation particle counter (CPC; allowing detection on the single particle level without any bias towards the chemical nature of the particle) or Faraday cup detector or an electrostatic nanoparticle sampler for subsequent investigations (AFM, EM, DotBlot etc.). This instrument is called macroIMS (ion mobility spectrometry), a.k.a. gas-phase electrophoretic mobility macromolecular analyzer (GEMMA) or scanning mobility particle sizer (SMPS). The second device is a custom-built device with an additional nDMA run in parallel (PDMA, parallel DMA) allowing the simultaneous size monitoring in an analytical DMA and after size-separation in a "so-called" preparative DMA the parallel collection of size-selected bionanoparticle fractions.

3 Results and Discussion

The characterization of viruses as human rhino virus, selected VLPs, vaccine particles, liposomes, polysaccharides, and VLP-antibody fragment complexes by means of nES connected to a DMA and a CPC will be demonstrated. Based on the determined sizes of the spherical bionanoparticles the molecular mass will be calculated via a compound-class-dependent correlation plot and evaluated. Furthermore the *off-line* combination of SEC will be demonstrated for vaccines. Size-separated bionanoparticles were collected on different surfaces (e.g. mica, copper grid or nitrocellulose) for subsequent analysis. This will be also shown with PDMA approach. Finally, first results on a radically new hyphenation technique, namely CE-naonES-DMA-CPC will be given.

4 Conclusions

The presented approach and data will demonstrate the closing of the gap between ESI QRTOF mass spectrometry and aerosol micrometer particle physics as well as a new hyphenation technique – electrophoresis in the liquid and gas phase.

Acknowledgements

This work was supported by Austrian Science Foundation (TRP29 and P25749-B20).

References

[1] Bacher, G., et al., *J. Mass Spectrom.* 2001, *6*, 1038-1052.

[2] Havlik, M., et al., *Analyst* 2014, *139*, 1412-1419.
[3] Weiss V., et al., *Anal. Chim. Acta* 2014, *841*, 91-98.

THE STUDY OF ULTRASMALL SAMPLES BY FAST CAPILLARY ELECTROPHORESIS-MASS SPECTROMETRY

Frank-Michael Matysik, Jonas Mark, Marco Grundmann, Sven Kochmann, Andrea Beutner

University of Regensburg, Institute for Analytical Chemistry, Chemo- and Biosensors, Regensburg, Germany
frank-michael.matysik@chemie.uni-r.de

Summary
The hyphenation of capillary electrophoresis (CE) with mass spectrometry (MS) is an attractive tool of instrumental analysis. However, the combination of conventional CE systems with MS using sheath-liquid ESI sprayers is usually associated with the implementation of rather long capillaries (longer than or equal to 60 cm). Consequently, the window of migration times is typically in the range of 5-10 min or even longer. In order to speed up CE-MS separations much shorter capillaries and rather high separation voltages should be applied.

We present a novel instrumental approach for fast CE-MS measurements in the time scale of seconds; in addition ultrasmall samples in the nanolitre range can be studied. Samples are handled by means of a microprocessor controlled injection system with an integrated capillary. The injection capillary is moved out of the injection cell for sample take-up from a microenvironment, and after repositioning it is facing the fixed inlet of a short separation capillary (15 cm in length), then a small sample plug is injected onto the inlet of the separation capillary. In this way separations of catecholamines could be carried out in less than 12 s.

The capability of handling samples in the nanolitre range allows for a rapid pre-concentration protocol based on the simple volume reduction by evaporation from µL to nL sample volumes. The efficient usage of available sample is of key importance in many bioanalytical questions for which CE-MS is an attractive method. The main advantage of separations in short capillaries is the dramatical decrease in migration times which is an important requirement for high-throughput analysis in the life sciences.

A very recent development from our laboratory is the concept of two-dimensional separations of ionic species by combining ion chromatography – fast capillary electrophoresis – mass spectrometry. This novel approach of ion analysis will also briefly be discussed.

TIME-RESOLVED CRYO-ELECTRON MICROSCOPY OF MACROMOLECULES

Tanvir Shaikh

CEITEC, Brno, Czech Republic

Summary

The goal of structural biology is to understand the function of macromolecules from their three-dimensional organization. Using cryo-electron microscopy (cryoEM), one pursues this goal by imaging hundreds or thousands up to a few million macromolecules, determining their relative orientations, and computing a three-dimensional reconstruction. Typically, static structures are studied, or perhaps an ensemble of structures populated under steady-state conditions. One area that has so far been explored little using cryoEM has been the dynamics of macromolecules. Since individual molecules can be imaged, cryoEM is well-suited to this task, although a particular state must be present in thousands of copies in order to obtain a 3D reconstruction. With the goal of implementing time-resolved cryoEM, we have developed a microfluidic device which mixes two components, sprays the mixture onto an EM grid, and then plunges the grid into cryogen. Here I describe results of such experiments mixing ribosomal subunits. I will also contrast different time-resolved methods in EM, and describe future development.

IMPROVING ENANTIOSELECTIVITY AND RESOLUTION IN CEC AND NANO-LC: RECENT RESULTS

Salvatore Fanali

Institute of Chemical Methodologies, Italian National Research Council (C.N.R.), Monterotondo, Italy

Summary

The separation of chiral compounds has been demonstrated a long time ago with the interesting work by Pasteur related to the separation of tartrate isomers. Since that time efforts have been devoted by a large number of researchers to develop theory, new methodologies, new chiral stationary phases, new instrumentation etc.

The analysis of enantiomers is an important topic in different areas including agrochemical, environment, biochemistry, pharmaceutical where new methodology offering high enantioresolution and selectivity is often required.

Chiral separation has been obtained utilizing several techniques such as gas chromatography (GC), High-performance Liquid Chromatography (HPLC), electrophoresis etc. All of them were also successfully investigated in the

miniaturized format. These analytical techniques offer several advantages over the conventional ones, e.g., reduced consumption of both sample, mobile and stationary phase, reduced waste and costs. In addition they also offer high resolution and high efficiency allowing for fast separations.

Aim of this communication is to briefly illustrate the state of the art of chiral separation achieved by using CEC and nano-LC as well as the features of these two techniques. Examples of new chiral stationary phases (CSPs) applied in both techniques, packing procedure used, selection of optimal experimental conditions, coupling with mass spectrometry (MS) will also be illustrated.

THE VALUE OF BIOBANK PATIENT SAMPLES IN PROTEIN EXPRESSION STUDIES

György Marko-Varga[1,2]

[1]*Clinical Protein Science & Imaging Group, BioMedical Center, University of Lund, Lund, Sweden*

[2]*Dept. of Surgery, Tokyo Medical University, Tokyo, Japan*

Summary

Many of the modern approaches for studying disease compare steady state functions, such as repair, growth, and regulated gene expression within the various biological compartments organised by specialized function, be it mitochondria or blood vessels. The assignment of protein identities, which are linked to key biological mechanisms, which are associated with disease processes and disease progressions are an important area of this work. Today, the technology available for studying proteome expression and resolving exact protein and peptide identities in complex mixtures of biological samples allows global protein expression within cells, fluids, and tissue to be approached with confidence. This confidence is due in part to reproducible repetitive sampling and analysis technologies including robotics data acquisition and high level mass spectrometry including both laser-desorbtion and electrospray ionisation. The precision in defining differences between normal and diseased steady states is aided by the creation of compiled reference and master data sets and by new methods for multiplexing the analysis of samples in groups. The establishment of key representative reference proteome systems representing the dynamic changes in protein expression during disease will be vital to the interpretation of changes observed in specific samplings of disease states and specific cells obtained from these samples. The creation of reference databases of proteins linked to disease pathways will play an important role in furthering our understanding of the "proteome of disease". Examples will be given where protein expression patterns have been generated from compartments within tissue sections as well as clincal studies directed to drug action and Biomarker developments.

SEX DURING COMMUNISM. INTIMATE LIFE AND THE POWER OF EXPERTISE

Kateřina Lišková

Masaryk University, Brno, Czech Republic

Summary

Sexuality seems to be something innate and biological, as if it were around in the same form since the dawn of time. Research in the social sciences, however, has shown persuasively that what people perceive as sex, the ways in which they understand themselves as sexual beings and even sexual practices change across time and place. Moreover, sexuality might be perceived as the innermost part of ourselves but its forms and expressions are strongly culturally mediated. Sex is formed by society and, conversely, we can make sense of the broader social arrangements if we study sexuality.

What did, then, sex during communism look like? What was seen as normal and deviant? How did these perceptions change over time? On the case of Czechoslovakia between the years 1948 and 1989, I will show the ways in which sex changed in connection to the shifts in the regime and its priorities.

THE MONKEY KING AND PIGSY FERRYING THE PROTEOMIC SUTRAS INTO THE THIRD MILLENNIUM

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Summary

About fifty years of progress in "proteome" analysis, starting from primitive two-dimensional (2D) maps attempts in the early sixties of last century, will be here covered. The polar star in 2D mapping arose in 1975 with the classical paper by O'Farrell in J Biol. Chem. It became the compass for all proteome navigators. Perfection only came, though, with the introduction of immobilized pH gradients, which fixed the polypeptides spots in the 2D plane. Great impulse in proteome analysis came by introducing informatics tools and creating databases, among which Swiss Prot remains the site of excellence. Towards the end of the nineties, 2D chromatography, as epitomized by coupling strong cation exchangers with C_{18} resins, began to be a serious challenge to electrophoretic 2D mapping, although up to the present both techniques are still much in vogue and appear to give complementary results. Yet the migration of "proteomics" into the third millennium was only made possible by mass spectrometry (MS, the Monkey King!), which today represents the standard analytical tool in any lab dealing with proteomic analysis. Another major improvement has been the

introduction of combinatorial peptide ligand libraries (CPLL, Pigsy!), which, when properly used, enhance the visibility of low-abundance species by 3 to 4 orders of magnitude. Coupling the Monkey King with Pigsy permits to explore at least 8 orders of magnitude in dynamic range on any proteome.

HYDROPHILIC INTERACTION CHROMATOGRAPHY-THE STATE OF THE ART

David McCalley

Faculty of Health and Life Sciences, University of the West of England, Bristol, U.K.

Summary

Hydrophilic interaction chromatography (HILIC) is a technique that has attracted increasing interest over the last decade, with over 350 primary publications last year, compared with only a handful ten years ago. The method is very useful for the separation of polar and ionisable compounds which tend to have poor retention in reversed-phase (RP) separations. Alpert coined the name HILIC in 1990, but the technique was in existence for many years previously, and may even originate in the early work of Martin and James in the 1940s. HILIC uses stationary phases similar to those in normal phase (NP) but with eluents comparable to those used in RP separations. Acetonitrile (>60%,v/v) together with aqueous buffer (>2.5%, v/v) is a typical eluent. HILIC is distinct from NP-LC where strenuous attempts are made to exclude water from the mobile phase. Partition of the solute between a layer of water held on the surface of the polar stationary phase and the bulk mobile phase appears to be a major mechanism. Evidence for the water layer exists from both experimental studies and computer simulations. The solute distribute coefficient D between octanol and water thus serves as a guide to the suitability of a particular solute for analysis using HILIC. However, selectivity differences which exist between different stationary phases, show that the column cannot function merely as an inert support for the water involved in a partitioning process. Adsorption onto polar column groups, ionic retention and even RP retention can also contribute. This lecture will discuss evidence for these various interactions.

Despite the complex separation mechanism, HILIC is rather easy to implement in practice. The selection of stationary and mobile phases will be considered, along with the best strategies that should be employed in order to manipulate the selectivity of a separation. The kinetics of HILIC separations will also be discussed and how its behaviour differs somewhat from expectations. Examples of different HILIC analyses will be given and the advantages and limitations of HILIC will be emphasised.

MICROFLUIDICS COUPLED WITH MASS SPECTROMETRY FOR ONLINE MONITORING OF DYNAMIC BIOLOGICAL PROCESSES

Ryan T. Kelly

Pacific Northwest National Laboratory, Richland, USA

Summary

Active microfluidic devices with integrated pneumatic microvalves enable automated, multistep biochemical analyses to be performed using subnanoliter volumes of sample and reagents. The control provided by the microvalves makes it possible to combine and mix reagents with high temporal resolution and incubate those reagents for variable durations to follow reactions over time, with timeframes ranging from seconds to hours. Importantly, the devices can incorporate integrated electrospray sources to enable sensitive, label-free and information-rich monitoring of reactions by mass spectrometry (MS). We will present the use of these integrated microfluidic devices to perform on-chip proteolytic digestion with MS detection as well as determining enzyme kinetics and protein-ligand interactions in both droplet-based and droplet-free platforms. Approaches to separate the reaction products by microchip electrophoresis prior to MS introduction will also be described.

HARSH ENVIRONMENT CAPILLARY ELECTROPHORESIS

Mihkel Kaljurand

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Summary

Deploying capillary electropherographs outside of the typical laboratory setting means placing complex equipment in diverse environments. These range from volcanoes, battlefields, hazardous urban sites, and ocean depths, to outer space and other rugged locales. Building capillary electrophoresis instrumentation to withstand the rigors of such harsh and remote environments poses unique technological challenges to engineering design and science objective planning. Stringent operational requirements for power, size and durability must all be met while achieving the goals of the scientific mission.

This talk will focus on recent developments in portable capillary electrophoresis instrument design. By applying sound engineering principles, many research groups have reported design innovations in systems that can be successfully deployed to the remote hazardous waste sites and even the planet Mars.

EVOLUTION OF A MICROFLUIDIC LC/MS SYSTEM - FUNDAMENTAL TECHNOLOGY TO A COMPLETED SYSTEM

Geoff Gerhardt

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Summary

Microscale LC offers significant advantages, particularly when coupled to a MS. Significant sensitivity gains can be realized when using microscale LC/MS (i.e. column i.d. <300um) versus analytical scale LC/MS (i.e. column i.d. >1mm). Unfortunately, microscale LC/MS systems lack the robustness and ease-of-use of analytical-scale systems. Typically plumbed with fragile fused-silica tubing using fine-tipped, narrow-bore glass electrospray needles, assembling and maintaining microscale LC/MS systems can be a tedious process. This presentation will describe a multi-year project to create a robust and easy-to-use microscale LC/MS system. Development of an integrated microscale LC consumable device consisting of a high-pressure ceramic microfluidic system, an integrated robust ESI emitter, temperature control and EEPROM for storing usage data. Also, a simple cartridge interface will be described that creates all the fluidic, gas and electronic connections to the device with a very simple user interface. In addition to describing the basic elements of this novel system, data will be showing illustrating the chromatographic and MS performance compared to more traditional analytical-scale LC/MS systems.

A LIGHT AT THE END OF THE TUNNEL FOR THE COMPREHENSIVE COMPOUND IDENTIFICATION IN UNTARGETED METABOLOMICS

Robert Mistrik

HighChem Ltd., Bratislava, Slovakia

Summary

Despite the increasing availability of modern high resolution mass spectrometers, untargeted metabolomics is hindered by an inability to identify thousands of observed components effectively. We will present an advanced computational and database framework leading to the much anticipated increase in mass spectral coverage of the metabolome, taking into account all the important experimental and calculated information necessary for efficient and reliable identifications. The resulting spectral space serves as a unique resource for the identification of unknowns even if reference spectra are not available, providing a major benefit for the metabolomics' community.

cHiPLC: MICROFLUIDIC COMPONENTS FOR HPLC

Don W. Arnold

Eksigent, Redwood City, CA, USA

Summary

In this presentation, we will describe a series of nanoLC-MS and microLC-MS proteomics results enabled by new microfluidic technologies, protocols and methods developed in our labs. We will discuss high-pressure microfluidic tools, zero-dead volume connectors, state-of-the-art nanoscale and microscale fluid delivery systems, new microfluidic workflows, integrated microfluidic sample preparation tools. The discussion will briefly describe the technologies and continue onto discuss a series of nanoLC-MS and microLC-MS results discussion that illustrate how they enable full workflow solutions that deliver sample-to-answer with the precision required to carry out the next generation of proteomics experiments.

SUB-2 µM SILICA PARTICLES WITH INTERNAL MACROPORES: DO WE NEED ANOTHER PARTICLE TYPE IN CAPILLARY SEPARATIONS?

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Summary

A new type of chromatographic particle has recently been synthesized at Indiana University (USA): its size is consistent with the use in UHPLC; the material has high porosity (~200 m^2/g) and features 100-nm interconnected macropores throughout its volume; and its surface can be functionalized to fit different modes of chromatography, including bioaffinity separations. After a brief physical characterization of these novel materials, they were derivatized with several types of lectins and evaluated as preconcentration materials for oncologically interesting glycoproteins isolated from human blood serum. The precolumn fabricated for immobilized lectins featured binding capacities of more than 10-fold greater than the conventional silica-based materials. More recently, we have evaluated performance of longer capillaries filled with these small particles for low-MW solutes using high pressures and low-dead-volume electrochemical detector (work done at the University of North Carolina). Very high column efficiencies have been obtained, corresponding to reduced plate-height values as low as 1.5. The column materials alternative to silica

are also being explored, while preparative hydrodynamic chromatography has been utilized to obtain particle fractions with a narrow particle size distribution.

Abstracts of oral presentations – CECE Junior

LA-ICP-MS AS A TOOL FOR ELEMENTAL MAPPING

Tereza Warchilová[1,2], Tomáš Vaculovič[1,2], Zuzana Čadková[3], Vítězslav Otruba[1], Jiřina Száková[4], Viktor Kanický[1,2]

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[2]Central European Institute of Technology, Masaryk University, Brno, Czech Republic
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Summary
Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) is becoming very favorite technique for direct solid sample analysis in analytical chemistry. At present, it represents one of the most sensitive techniques for the imaging of metals, metalloids (Se) and nonmetals (P, S, C). Imaging of these elements was applied to a wide range of biological samples (brain slices, plant tissues, bones, teeth). The major advantages of this technology are no or little sample preparation, possibility of minor and trace element analysis, high sensitivity and impressive lateral resolution which enables detection of small material defects. Elemental imaging by means of LA-ICP-MS of tapeworm cuts and accumulation of Pb in the tissues will be discussed.

1 Introduction

In recent years there has been increasing interest in the interrelationship between parasitism and pollution. It was proved that if an organism is exposed to a specific amount of contaminant (heavy metals – Pb, Cd) a certain portion of this dose could be accumulated by inner parasite most effectively than by the host tissues [1, 2]. More investigations are necessary to decide whether or not intestinal parasites are able to affect metal levels of host tissues. Obviously, the concentrations of risk elements in animal tissues (liver, kidney, lung, blood, hair) of the affected animals are determined in element contaminated areas. Tapeworms are more abundant in terrestrial mammals than acanthocephalans and thus potentially more useful in attempts toward passive as well as active biomonitoring. Very common animal *Rattus norvegicus* and their common tapeworm *Hymenolepis Diminuta* were selected for the experiment. Optimization of laser ablation parameters (repetition rate, fluence, laser spot diameter, scan speed) enables elemental imaging of proglottid *Hymenolepis diminuta* cross

section. 2D elemental maps indicate the inner organ (structure) of tapeworm proglottid which accumulates the heavy metals more effectively than others.

2 Experimental

2.1 Tapeworm cross section preparation

Two groups of male Wistar rats were inoculated by *Hymenolepis Diminuta* larvae and kept under standardized conditions for 6 weeks. One group was fed a dose of lead nitrate ones per week. Second group was control. After Pb-exposure, experimental animals were sedated and euthanized. Tapeworm tissues were fixed in solution of boiling 4% formaldehyde, dehydrated, cut in individual sections and embedded in paraffin wax. Mictrotom was used for 40 μm thin slices preparation. Tapeworm cross sections were fixed on adhesion microscope slides. The identical procedure was used in the case of sheep tapeworm *Moniezia Expansa*.

2.2 LA-ICP-MS instrumentation

Elemental mapping was performed on LA-ICP-MS instrumentation which consists of Nd:YAG laser ablation system UP213 (New Wave Research, Inc., ESI, Fremont, CA, USA) and an ICP-MS apparatus Agilent 7500CE (Agilent, Japan). The ablation device is equipped with XYZ-stages which enables sample movement before and during analysis. Optimization of LA-ICP-MS conditions (sampling depth, gas flow rates, electrostatic lenses voltages of the MS) was carried out using was carried out using the glass reference material NIST SRM 612. Optimization of laser ablation parameters (laser beam diameter, laser beam fluence, scan speed) was optimized using the tapeworm cut.

3 Results and Discussion

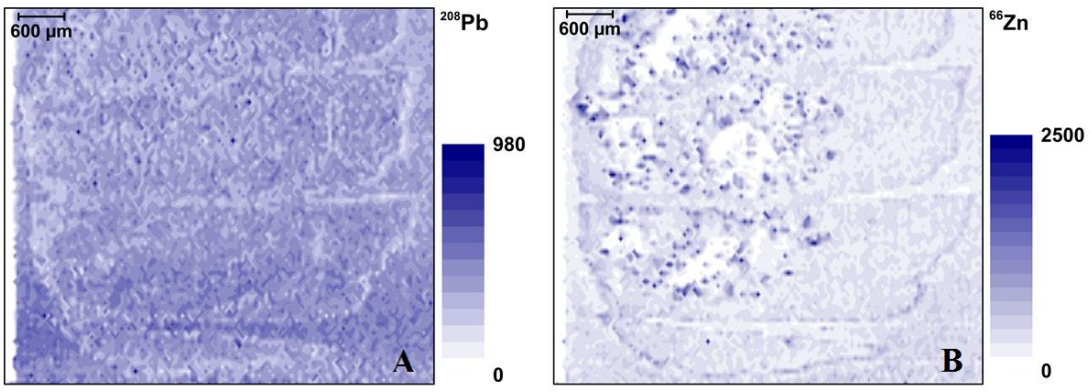

Fig. 1. 2D intesity maps of ^{208}Pb and ^{66}Zn (*Moniezia Expansa* from natural affected host).

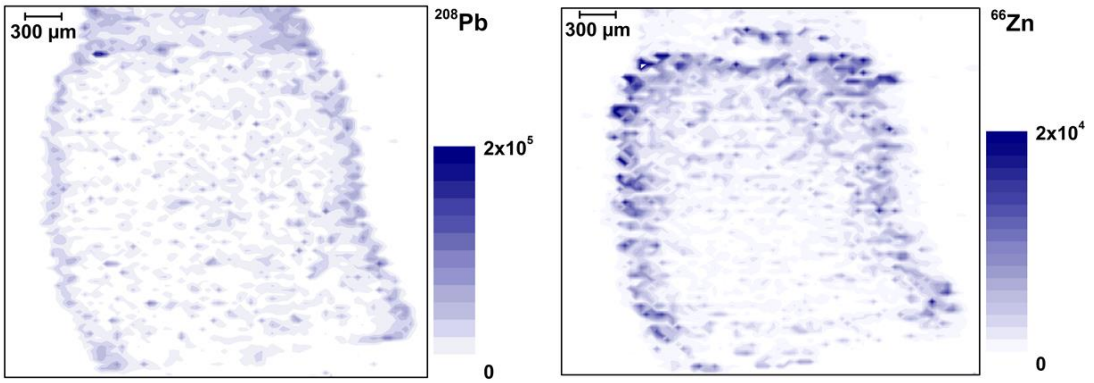

Fig. 2. 2D intesity maps of ^{208}Pb and ^{66}Zn (*Hymenolepis Diminuta* from Pb-affected rat).

4 Conclusions

Two kinds of intestine parasites were analyzed by means of LA-ICP-MS – *M. Expansa* from natural affected sheep and *H. diminuta* from experimentally affected rat. Laboratory rats were divided into two groups. First group was fed a dose $Pb(C_2H_3O_2)_2$ and the second was control. It was found out that Pb, Zn and Cu are accumulated at the edge of the cut of the proglottid more effectively than in inside parts. Intensities of Pb signals achieved with *M. Expansa* (from natural host) analyses are more than 100x lower than in *H. diminuta* case. Proper quantification strategy application and improvement of resolution value enable to calculate the concentration of metal in tapeworm organs. Subsequently this experiment will allow to describe in detail the risk element pathway and translocations in animal-parasite interactions.

Acknowledgement

This work was supported by the Czech Science Foundation project (GA13-18154S), CEITEC - Central European Institute of Technology (CZ.1.05/1.1.00/02.0068), European Regional Development Fund and Student Project Grant at MU (specific research, rector's program) – Category A (MUNI/A/0992/2009).

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THIOL-ENE-BASED MONOLITHIC MICROREACTORS

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1 Introduction

Thiol-enes are UV-curable copolymers prepared from a mixture of thiol- and allyl-group containing monomers. Radical reaction between thiolic and allylic group shows characteristics of the so called "click" chemistry – a group of strong, rapid and specific synthetic reactions requiring only mild conditions. Curing is fast, often initiated without the need for addition of photoinitiator. Properties of the resulting material are influenced by the ratio of the functional groups present in the monomer mixture [1].

Mixing thiol-ene with porogenic solvents allows for fast and simple formation of UV-curable monoliths, in which porosity is influenced by the amount and nature of the added solvent. Dispersions of thiol-ene monomer mixture in porogen are easily prepared and cured to form a dense network of interconnected polymer beads.

The use of the so-called off-stoichiometry thiol-ene chemistry allows for easy surface functionalization and biomolecule immobilization. Properties of the material allow for UV-induced reaction of allyl-group in ene-excess thiol-enes with thiol-containing molecules, e. g. introduction of amino-group by aminoethanethiol [2]. Classic immobilization reactions on thiol-groups can be realized on thiol-excess polymer [3].

2 Experimental

In this work, structures were micromilled in PMMA and replicated in PDMS. The resulting PDMS replicas were used as molds for the thiol-ene chips (40% excess thiol). The thiol-ene slabs (2 mm thick) were cured by exposition to collimated UV-light (4 seconds, 23mW/cm^2 at 365 nm). The thiol-ene slabs were bonded together by exposing the parts placed in conformal contact to UV light.

A thiol-ene emulsion (40% excess ene thiol-ene) was prepared by mixing thoroughly 1 part of thiol-ene to 4 parts of methanol. The mixture was introduced inside the thiol-ene microchannel. Areas of the channel where monolith was not supposed to form were tightly covered to shield them from UV.

After curing with a collimated UV-light, the unreacted mixture was washed out of the channel. Further surface modification of the monolith via thiol-ene click chemistry is possible.

3 Results and Discussion

The monolith mixtures, using methanol as porogen, form emulsions (or dispersions) of thiol-ene droplets in a continuous phase of solvent. Stirring using a magnetic stir bar causes formation of micrometer-sized thiol-ene droplets. When exposed to UV-light, the droplets solidify into bead-like structures which are fused together forming a monolithic network of interconnected beads.

Using chip made of complimentary ratios of thiol- and allyl-monomers allowed for reaction between the beads and the wall of the channel initiated by UV-curing. This caused a fusion of the wall and the monolith, anchoring it strongly in the channel and preventing it from "bleeding".

Although technically a monolith, structures resembled more closely a packed column. This was a reason for relatively high back pressure formed at the monolith which is caused by the small size of cavities.

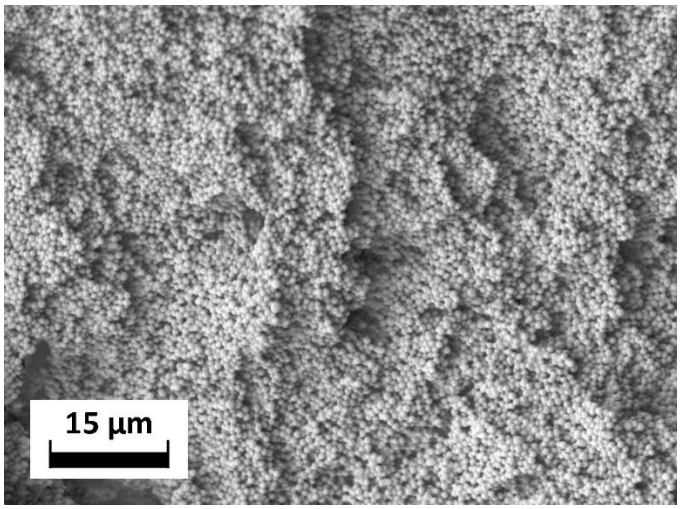

Fig. 1 SEM picture of monolith formed from 80% methanol mixture (2000x)

4 Conclusion

High variability in properties of thiol-enes makes them ideal material for microfluidic reactors. Surface functionalization can be carried using functional groups already present in the material and can be easily patterned thanks to UV-initiation.

As the character of porogenic solvent, thiol-ene concentration, use of detergents, speed of stirring, etc., have influence on formation and properties of the resulting monolith, optimization of the procedure for its intended application is necessary.

Acknowledgements

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POLYELECTROLYTE MULTILAYER COATINGS FOR THE SEPARATION OF PROTEINS BY CAPILLARY ELECTROPHORESIS: INFLUENCE OF POLYELECTROLYTE NATURE

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Summary
A broad library of polyelectrolyte multilayers was compared for the electrophoretic separation of protein mixtures under CE/MS compatible experimental conditions. Peak efficiency and migration time repeatability obtained for the separation of a mixture of five proteins were systematically determined for more than 20 couples of different polyelectrolytes.

1 Introduction

Capillary coatings are crucial for high-quality separation performance in capillary electrophoresis and capillary electrophoresis coupled to mass spectrometry analysis of proteins to prevent adsorption phenomena onto capillary wall and to increase the repeatability of the separations [1]. Until now, only some polyanion (PA) / polycation (PC) couples were studied in literature [2-4]. The present work aims to study a wide library of polyelectrolytes of different natures (polypeptide, polysaccharide, vinylic or allylic polymers, polyionene) as components in 5-layer systems for protein separation in MS-compatible background electrolyte.

2 Experimental

CE experiments were performed on a P/ACE MDQ system (Beckman, USA). UV detection was performed at 214 nm at 25°C. Untreated fused silica capillaries (Polymicro Technologies, USA) of 40 cm total length (29.6 cm to the detector) and 50 µm i.d. were used. PC and PA layers (5 layers ending with PC) were successively built using solutions at 3 g/L in 20mM HEPES buffer at pH 7.5 (1 bar, 7 min) with intermediate rinsing steps with HEPES buffer (1 bar, 3 min). Protein sample mixture was composed of lysozyme, β-lactoglobulin, RNAse A, myoglobin and trypsin

inhibitor at 1g/L in water [5]. Injection was performed at 30 mbar for 4 s. BGE was 0.5 M acetic acid, pH 2.5. Separation voltage was -30kV. The studied polycations were: poly(L-lysine) (Plys), poly(L-lysine,serine) 3:1 (P(lys,ser)), poly(L-lysine,tyrosine) 1:1 (P(lys,tyr)), Poly(ethyleneimine) (Pei), Diethylamino-ethyl-dextran (DEAED), poly(allylamine) (Paam), poly(diallyl dimethyl ammonium chloride) (PDADMA) and poly(dimethylaminoethyl methacrylate (Pdmaema). The studied polyanions were: poly(styrene sulfonate) (PSS), poly(methacrylic acid) (PMA), poly(acrylic acid) (PAA), poly(acrylic acid-co-acrylamide) ($Paa_{80}am_{20}$ and $Paa_{20}am_{80}$), poly(L-lysine citramide) (Plc), poly(L-glutamic acid) (Pglu), dextran sulfate (DS) and hyaluronic acid (Ha).

3 Results and Discussion

Peak efficiency and RSD on migration times obtained for the different polyelectrolyte multilayer systems are presented in Figure 1 with example of electropherograms.

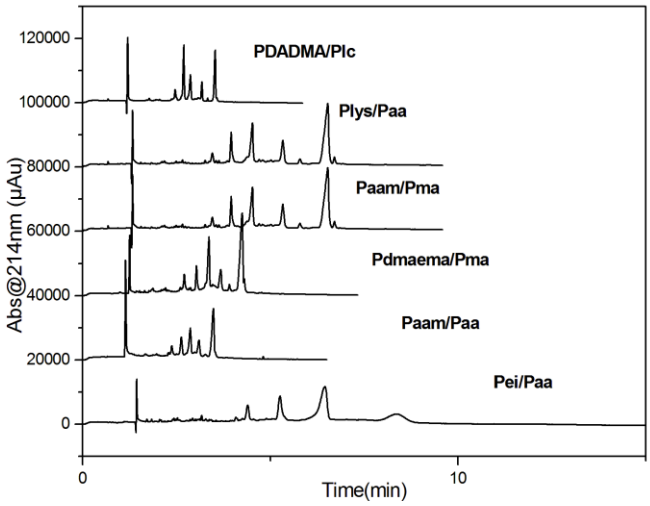

Fig. 1A. Typical electropherograms obtained for the test protein mixture on a few tested polyelectrolyte couples. Capillary length: 40cm (29.6cm to detection) ×50µm i.d; BGE: 0.5M Acetic Acid; V=-30KV Tinj= 4s, Pinj=30 mbar.

Separation efficiency (average on 5 proteins) varied from 103000 plate.m^{-1} for PDADMA-Plc system to 8000 plate.m^{-1} for P(lys,ser)-Ha system, showing the great importance of the choice of polyanion /polycation combination on the separation performances. The coating stability also varied significantly from very stable coatings with RSD < 1% to unstable coatings with RSD values above 20%. In the case of vinylic (Pdmaema) and allylic polycations (PDADMA and Paam), the more hydrophobic the polyanion partner, the more efficient the separation is (except Plc). In the case of Plys and derivatives, the more hydrophilic the polyanion partner, the more efficient the separation is. Pei polycation and Ha polyanion showed the worst results in terms of separation efficiency, whatever the partner of the opposite charge. The better results generally obtained when using Plc as the polyanion

could be explained by its particular structure which (i) is close to protein structure (amide bonds), (ii) has a lower charge density than other polyanions (as proteins) and (iii) carries hydro-philic hydroxyl groups that are known to limit protein adsorption. The number of polyelectrolyte layers and the ionic strength and the pH of the polymer solution have been studied in details and will be discussed.

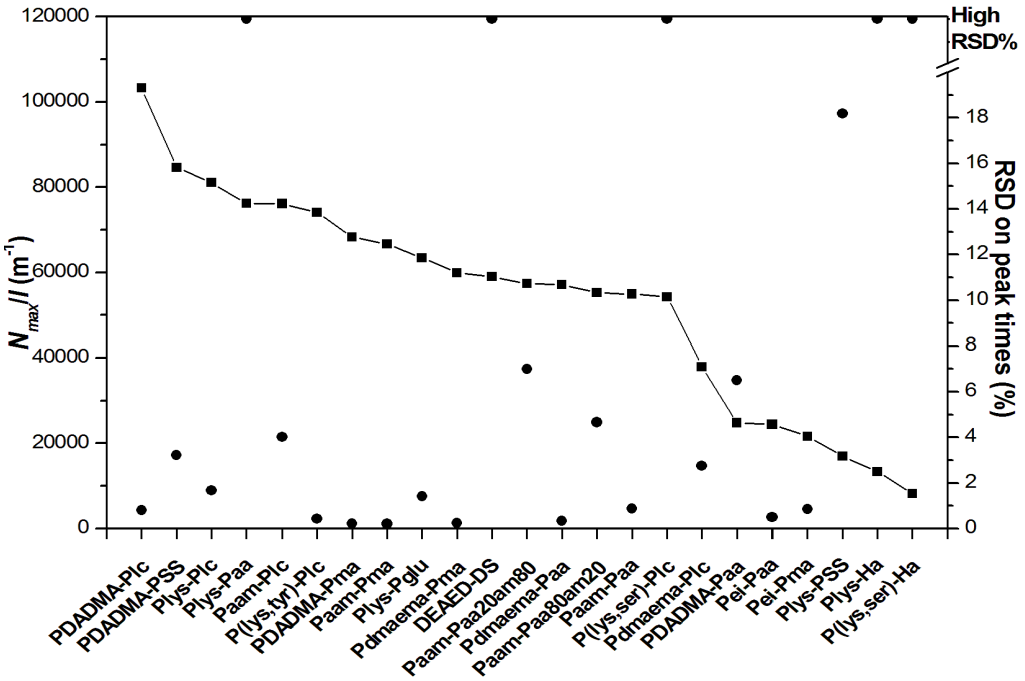

Fig. 1B. Peak efficiency (Nmax/l) and coating stability (RSD on peak migration times) for all tested 5-layer polyelectrolyte coatings.

4 Conclusions

Best results in terms of peak efficiency and repeatability were obtained with PDADMA-Plc system (5 layers). The impact of a supplementary final layer on the EOF and separation performances will be studied in the future to improve our knowledge on polyelectrolyte multilayer-based coated capillaries.

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IN VITRO RNA RELEASE OF A HUMAN RHINOVIRUS FOLLOWED VIA A MOLECULAR BEACON AND CHIP ELECTROPHORESIS

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Summary
With the current work we present an *in vitro* assay to follow the RNA release out of intact particles of a human Rhinovirus via molecular beacons (fluorescent probes able to detect a complementary oligonucleotide sequence) and chip electrophoresis. Hence, a fast, sensitive and easy-applicable method to detect infectious virus material is introduced. Additionally, by setting up a corresponding assay we will be able to investigate factors of viral uncoating.

1 Introduction

Human Rhinoviruses (HRVs) are non-enveloped particles of approx. 30 nm diameter [1, 2]. They are composed of four viral proteins (VP1 – 4), 60 copies each, forming the virus capsid and a single stranded, positive sense RNA genome of approx. 7.1 kbases attached to a single copy of VPg [3]. To date, more than 160 serotypes have been characterized, which according to their receptor specificity can be classified to a minor, a major as well as into the HRV-C group. The receptor for HRV-C group viruses is still under investigation [4]. Work presented in the current presentation has been carried out with HRV serotype 2 (HRV2), a minor group virus, which binds to members of the low-density-lipoprotein receptor family for cell entry.

Following receptor mediated endocytosis, the viral RNA genome is shuttled across the membrane of late endosomes to reach the cytosol of an infected cell and to initiate HRV proliferation. *In vitro*, several stages of virus uncoating (conformational changes of the virus capsid related to the release of the RNA genome out of the protective proteinaceous viral shell) can be differentiated: Native virions (four viral proteins and RNA, particles sedimenting at 150S) first release VP4 to allow for capsid permeability. Corresponding subviral particles (three viral proteins and RNA) sediment at 135S. In a second step, the viral RNA is released and empty capsids (three viral proteins, particles sedimenting at 80S) are obtained [5]. HRV2 uncoating can either by triggered by acidification (pH < 6.0) or sample heating (10 min to 56 °C). Depending on several factors, like presence of divalent cations, preferentially, subviral particles or empty viral capsids are formed [6, 7].

In a series of publications we reported the electrophoretic analysis of fluorescently labeled HRV2 in the chip format [8, 9]. Additionally, we were able (i) to follow the attachment of virions to liposomes [10, 11], vesicles mimicking cell membranes, and (ii) to detect changes in the liposome membrane permeability upon

virus uncoating [12, 13]. We now demonstrate the applicability of molecular beacons (MBs), short, single-stranded oligonucleotide sequences, to follow the release of viral RNA in combination with chip electrophoresis. MBs are modified by a fluorophore/quencher pair at the oligonucleotide ends. Under favourable conditions (e.g. ionic strength), proximal bases form a helix structure (stem) leading to spatial proximity between the fluorophore/quencher pair: No fluorescence (FL) is recorded. In contrast, if a corresponding target sequence is present, oligonucleotides not involved in stem formation (i.e. the loop) attach to the target: The helix structure of the stem is lost and FL is recorded [14].

2 Experimental

2.1 Biological material

HRV2 was prepared and its concentration and infectivity was assessed as described [15]. Oligonucleotides were obtained from Integrated DNA Technologies (Leuven, Belgium). The MB sequence (Atto 633 - TCGCACAAAA+GCAAA+TCA+TACG+GAGGGATGCGA - Iowa Black RQ-Sp quencher) targeted a region close the 3' end. Several nucleotides were LNA modified (marked by '+'). A complementary oligonucleotide (elongated by eight nucleotides at each end) was employed as positive control.

2.2 Instrumentation

Chip electrophoresis was carried out on an Agilent 2100 Bioanalyzer instrument (Waldbronn, Germany) employing commercially available, single-use chips in the presence of electroosmosis [8, 9] and at T = 22.5 ± 1.5°C. Sodium borate, pH 8.5, including Trolox (total ionic strength, I, of approx. 40 mM) was employed as background electrolyte (BGE).

2.3 Sample preparation

Samples contained 40 nM MB as well as 2.5 µM Atto 488 and 3.3 µM Atto 495 as internal standards. Positive control target concentrations of up to 400 nM were employed. Signals for HRV2 samples corresponded to tissue culture infective dose ($TCID_{50}$) values of approx. 3×10^{10} / mL. Sample heating was for 15 min to 56°C to trigger RNA release. Alternatively, samples were acidified to pH 5.

3 Results and Discussion

3.1 Chip electrophoresis of MBs

Initial experiments had been carried out with a different MB to investigate the behaviour of MBs in dependance of I of the BGE. An increase of I lead to a reduction of the observed electroosmotic flow as well as to a concomitant higher degree of closed hairpin formation of MBs (formation of the stem double helix), i.e. less FL was recorded. An enzymatic digest of the MB allowed an estimate of the maximum possible FL gain under finally chosen BGE conditions. Application of divalent cations (e.g. Mg^{2+}) known to stabilize the MB stem was omitted as such additives were found to stabilize viral uncoating intermediates [7].

3.2 Impact of BGE additives on the FL response of the MB

Subsequent experiments targeted the MB reactivity in the presence of a positive control oligonucleotide. With increasing concentration of the positive control a concomittant increase of FL was recorded (Figure 1A). No such increase was detected for mixing of the MBs with a negative control oligonucleotide. In a second step it was tried to increase the FL signal obtained after the conformational change of the MB from the closed to the open hairpin formation (no spatial proximity between the fluorophore and the quencher in the latter case). As described, addition of additives is known to reduce blinking and photobleaching of fluorophores [16]. We therefore investigated the impact of Trolox addition on the obtained signal. Indeed an approx. twofold FL increase was detected. Additional experiments with ascorbic acid did not yield any increase in FL.

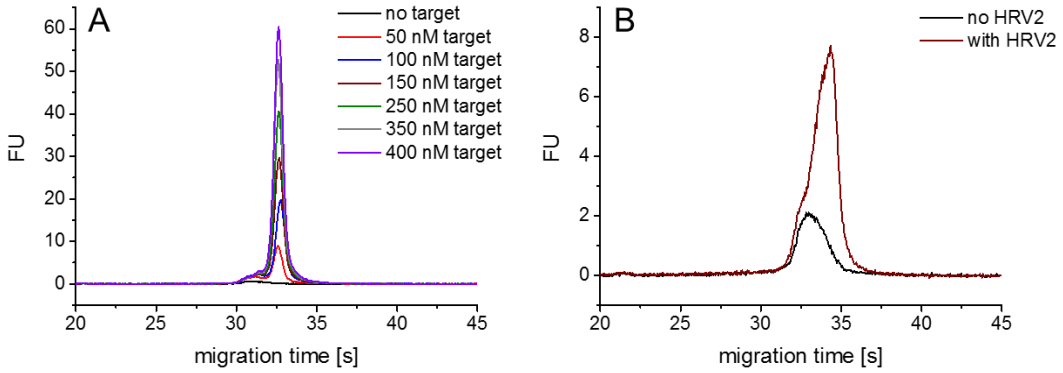

Fig. 1. FL signals resulting from the 'positive control oligonucleotide/MB' complex at indicated target concentrations (A). Signal obtained for the 'viral RNA/MB' complex (B).

3.3 Detecting HRV2 RNA release via MBs and chip electrophoresis

Following our previous experiments with positive control oligonucleotides we mixed the MB with HRV2 and triggered virus uncoating via sample heating and acidification, respectively. We found an additional desalting step of HRV2 preparations beneficial for the FL signal obtained from the MB binding to the released viral RNA genome. This effect is probably due to Mg^{2+} and other divalent cations still present within the original HRV2 sample reducing the amount of viral uncoating [7]. However, after this buffer exchange step we observed clear signals for the MB/RNA complex (Figure 1B for sample heating). To date it appears as if results obtained after sample heating yield a higher RNA amount accessible for MB binding than acidified samples (preliminary conclusion).

4 Conclusions

We present a fast, sensitive and easy-to-handle method to check liquid samples for the presence of infectious virus material. To date, we are able to detect virus concentrations down to $TCID_{50}$/mL levels of approx. 10^{10} infectious particles. Future

work (including sample preconcentration steps) is suggested to further lower this value.

Acknowledgement
Funding was provided by the Austrian Science Foundation (grant P25749-B20 to VUW).

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DEVELOPMENT OF A NOVEL RP-HPLC METHOD FOR THE DETERMINATION OF AMINO SUGARS IN SAMPLES OF ENVIRONMENTAL ORIGIN

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Summary
A novel HPLC method was developed to separate three amino sugars (D-glucosamine, D-galactosamine, D-mannosamine). The chromatographic separation was achieved using a monolithic reversed-phase column, pre-column derivatization with diethyl ethoxymethylenemalonate (DEMM) and diode-array detection (DAD). Mobile phase consisted of phosphate buffer and methanol. In environmental samples, we determined amino sugars at concentration levels of 1.166 to 2.380 μg/ml. The LOD and LOQ were calculated from calibration curves between 0.75 to 1.36 mg L^{-1} and from 2.29 to 4.13 mg L^{-1}.

1 Introduction

Amino sugars (AS) are widely distributed compounds in nature. They are synthesized directly in organism, what makes them the important indicators of diseases or disorders of the immune system. AS are components of biologically significant compounds, e.g., polysaccharides, glycoproteins, glycolipids or antibiotics [1].

In environmental terms, AS are important indicators of microorganism accumulation in soil. Due to their structure they contribute to the total organic carbon (TOC) and the total organic nitrogen (TON), which help in determining soil fertility and plant growth. In soil biomass, there are minimally 26 free AS, of which the TON represents between 5% to 12% and the TOC represents almost 3%. The largest proportion of AS in soil is to be found, in descending order, galactosamine (GalN), glucosamine (GlcN) and mannosamine (ManN) [2,3].

Determination of the AS is mostly achieved using a HILIC method [4] or RP-HPLC method with use of different derivatization reagents e.g., fluorenylmethyloxycarbonyl chloride (FMOC-Cl) [5] or o-phthaldialdehyde (OPA) [6].

The aim of this work was to develop a new method for separation of AS with the use of not frequently used reagent DEMM, and determination of AS in environmental samples.

2 Experimental

2.1 Materials and reagents

Standards D-glucosamine hydrochloride and D-galactosamine hydrochloride were purchased from Merck KGaA, D-mannosamine hydrochloride was purchased from

Sigma-Aldrich. The purity of standards was 99,8 – 99,9%. Derivatization reagent DEMM was purchased from Schuchardt OHG (Merck).

Environmental samples of humic acids (HA) were isolated by the modified IHSS fractionation at the Department of Analytical Chemistry, Comenius University, from peat (location Cerová hora) and soil (location Dunajská Streda).

2.2 Instrumentation and chromatographic conditions

A Merck-Hitachi LaChrom chromatographic system equipped with a L-7100 gradient pump, L-2000 autosampler, L-7450A DAD, L-7300 column termostat, and a D-7000 HSM software was used. The monolithic type of analytical column Chromolith Performance RP-18e (100 x 4.6 mm i.d.) with guard column Chromolith Guard Cartridges RP-18e (5 x 4.6 mm) and gradient elution with a mobile phase consisting of phosphate buffer and methanol were used for the separation of AS. The mobile phase was delivered at a flow rate of 1.0 mL min^{-1} and the eluent was evaluated at a wavelength of 280 nm. The system was operated at 40°C.

2.3 Acid hydrolysis of HA samples

Each HA sample was weighted to 50.0 mg and put in a glass ampoule, and 500 µL of 3 M hydrochlorid acid solution was added to each ampoule. The ampoules were sealed and kept for 24 h at 90°C, which resulted in releasing AS from biological complexes in humic matrix. Hydrolysed samples were centrifuged and derivatized with DEMM.

2.4 Derivatization of degradation products of HA samples

After centrifugation, each supernatant was transfered into a 25 mL volumetric flask, 10 mL of borax solution (0.1 M) and 100 µL of DEMM were added. Derivatization reaction was carried out in water bath during 60 min at 55°C. After derivatization, sample solutions were cooled, 2 mL of methanol were added and volumetric flasks were filled with borax solution.

3 Results and Discussion

As shown in following figures, there are six peaks instead of three, two for each amino sugar. We believe this effect can be attributed to anomerization of amino sugars, what is also suggested by other literature and publications. In this work, we focused on the optimalization of some separation conditions, e.g., the effect of the ionic strength of mobile phase and pH of phosphate buffer onto chromatographic resolution of analytes.

3.1 Optimalization of ionic strength of phosphate buffer

The ionic strength of phosphate buffer was altered by changing its concentration from 10 mmol L^{-1} to 50 mmol L^{-1}. As shown in Figure 1, the change of concentration has no effect on the retention of AS standards. Optimum concentration of phosphate buffer was chosen 10 mmol.L^{-1}.

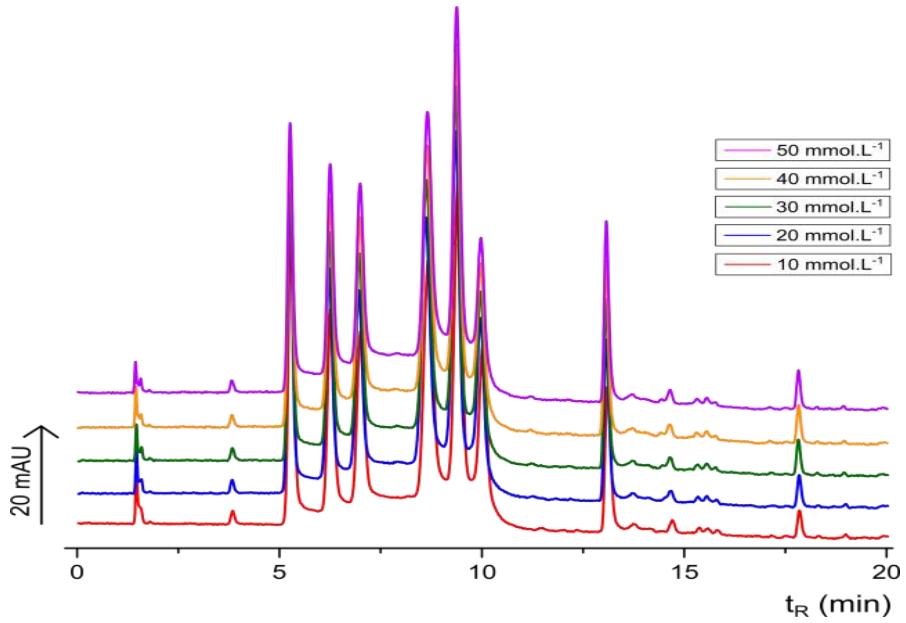

Fig. 1. Chromatographic profiles of AS standards under different concentrations of phosphate buffer.

3.2 Optimal pH of phosphate buffer

The pH of phosphate buffer was adjusted in an interval from 3 to 5 using 10 mmol L^{-1} phosphoric acid. As shown in Figure 2, the change of the pH has no effect on the retention of AS standards. The pH of phosphate buffer was adjusted to 3.60 as optimal value.

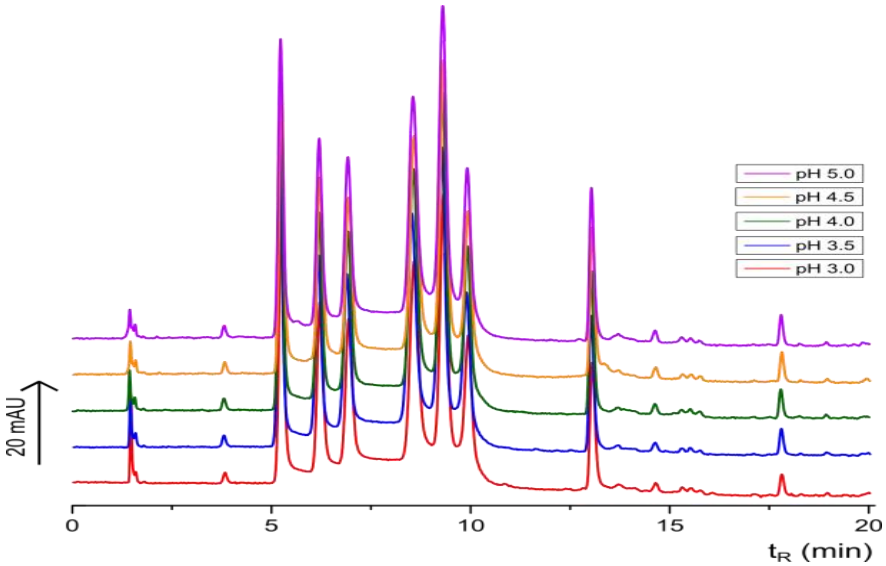

Fig. 2. Chromatographic profiles of AS standards under different pH of phosphate buffer.

3.3 Determination of AS in degradation products of HA samples

The suggested HPLC method was found effective for determination of two AS in degradation products of HA samples. Analysis results were evaluated with the calibration curve method and the method of standard addition.

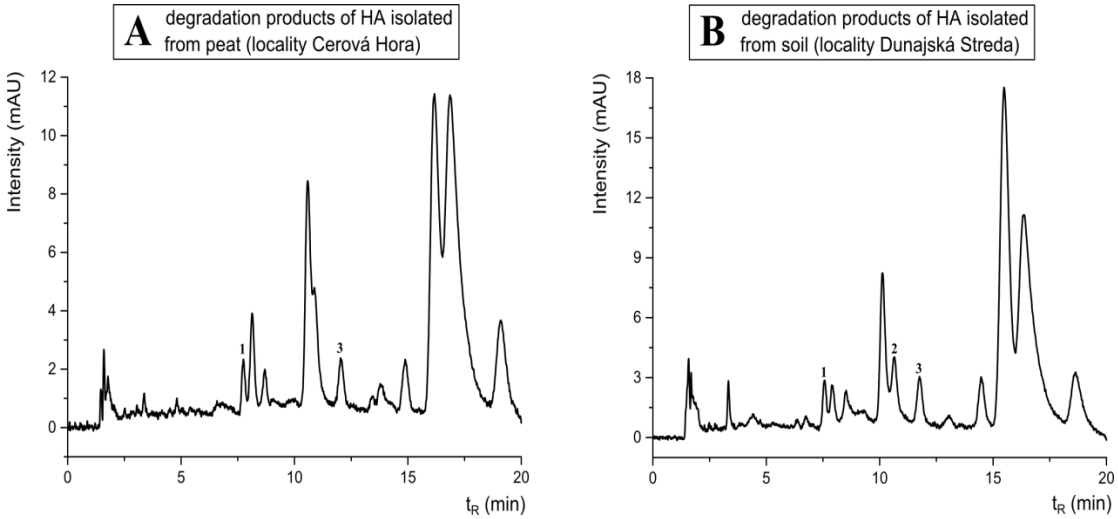

Fig. 3. Chromatographic profiles of degradation products of HA: 1-GalN 1, 2-GalN 2, 3-GlcN 1.

4 Conclusions

A novel HPLC method for determination of AS in samples of environmental origin was developed. In environmental samples, namely, degradation products of humic acids we determined two amino sugars at concentration levels of 1.166 to 2.380 µg/ml. The LOD and LOQ were calculated from calibration curves between 0.75 to 1.36 mg L^{-1} and from 2.29 to 4.13 mg L^{-1}.

Acknowledgement

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HPLC DETERMINATION OF METHIONINE, HOMOCYSTEINE AND CYSTEINE ENANTIOMERS IN SERUM OF PATIENTS AFTER STROKE

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Summary
Homocysteine is sulfur-containing amino acid that is naturally occurring in human body. It is produced from essential amino acid, methionine. Homocysteine can be remethylated back to methionine or converted to cysteine. The association between these amino acids evokes the need for their simultaneous determination. There are many studies focusing on achiral separations. In this work, chiral separations of homocysteine, cysteine and methionine enantiomers are presented. Their concentrations were estimated by using two-dimensional HPLC system with electrochemical detection. The amino acids were separated on achiral column and their enantiomers on Chirobiotic TAG column. The proposed method was applied to the analysis of human serum of healthy volunteers and patients after stroke.

1 Introduction

Elevated concentration of homocysteine has been associated with increased risk for cardiovascular and cerebrovascular disease [1]. A stroke occurs when the blood supply to part of the brain is interrupted or severely reduced, depriving brain tissue of oxygen and food. Within minutes, brain cells begin to die [2]. Aim of this study was to propose HPLC method for simultaneous determination of homocysteine, cysteine and methionine enantiomers which could be applied to analysis of serum samples and find out which homocysteine enantiomer participates in pathogenesis of the disease.

2 Experimental

Blood was obtained from 2 healthy elderly volunteers (1 man and 1 woman age 69 and 49) and 2 patients after stroke (2 men age 68 and 52). Serum samples were pre-treated according to Garaiova et al. [3]. The supernatant (20 µL) was injected into the HPLC system. The HPLC system consisted of an isocratic pump and electrochemical detector (Coulochem II, ESA). Detector was composed of guard cell Model 5020 and analytical cell Model 5010A (ESA). Achiral separations were performed on Purospher RP-18 endcapped 250-4 mm (5 µm) used together with a pre-column Purospher STAR RP-18e (5 µm) (Merck, Germany). Chiral separations were performed on Chirobiotic TAG 250-4.6 mm (5 µm) (ASTEC, USA).

3 Results and Discussion

The results show that patients after stroke had higher level of L-homocysteine in comparison to healthy volunteers (Table 1). We can assume that there is an association between homocysteine and stroke. We have not detected the presence of its D-enantiomer (< LOD 0.8 μg mL^{-1}).

Table 1. Concentration of amino acids in serum of healthy volunteers (A) and patients after stroke (B)

A	Volunteer 1	Volunteer 2
Total serum thiols	Mean ± SD	Mean ± SD
L-HCy	3.0 ± 0.2	3.2 ± 0.3
L-Cys	13.3 ± 0.2	10.4 ± 0.4
L-Met	3.0 ± 0.1	2.4 ± 0.4

B	Patient 1	Patient 2
Total serum thiols	Mean ± SD	Mean ± SD
L-HCy	8.3 ± 1.4	7.9 ± 0.5
L-Cys	15.0 ± 0.0	12.6 ± 0.3
L-Met	5.2 ± 0.7	1.3 ± 0.1

Data are presented as mean and standard deviations [μg mL^{-1}]
HCy – Homocysteine, Cys – Cysteine, Met – Methionine

4 Conclusions

In this work, the HPLC method was proposed for simultaneous determination of methionine, homocysteine and cysteine enantiomers by using two columns in on line system. The proposed method was applied to the analysis of human serum of healthy volunteers and patients after stroke. The first results show that only L-homocysteine participates in pathogenesis of the disease. However, it needs to be confirmed in a larger study involving more patients.

Acknowledgement

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CAPILLARY ELECTROPHORETIC ANALYSIS OF SINGLE BREATH - POSSIBLE OR NOT?

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Summary
The potential of capillary electrophoresis (CE) to analyze exhaled breath condensate (EBC) from a single exhalation is shown. A low cost, miniature, portable sampling device for EBC collection was developed for repeatable EBC sampling and has a significant implication for the future applicability of the EBC as fast and non-invasive diagnostic tool. CE with contactless conductivity detection is used as a sensitive technique to detect various nitrosative stress markers in EBC.

1 Introduction

Exhaled breath condensate (EBC) is a promising diagnostic body fluid that can be applied in lung respiratory research and diagnosis. An ultimate goal of breath research would be to diagnose lung condition from a single exhalation. On the way towards this goal, we have developed a low cost, simple and portable miniature sampling device for collection of EBC from single exhalation. For measuring of the ionic content of EBC (including markers of nitrosative stress) we have used a purpose-built capillary electrophoresis (CE) instrument with capacitively coupled contactless conductivity detector (C^4D).

2 Experimental

2.1 Electrophoretic and detection system, injection, electrolytes

A purpose-built CE instrument with C^4D detector [1] was employed for all electrophoretic separations. The separation voltages of – 15 kV (cations) and +15 kV (anions) were used. Fused-silica capillaries (50 µm id, 360 µm od, 50 cm total length and 20 cm effective length, Polymicro Technologies, Phoenix, AZ, USA) were used for the separation. Standards and EBC samples were injected hydrodynamically at 15 cm for 60 s. The optimized BGE composition used in this work was 60 mmol/l MES, 60 mmol/l HIS (L-Histidine), 30 µmol/l CTAB and 2 mmol/l 18-crown-6 at pH 6.

2.2 Sampler for collection of EBC

The sampler for collection of EBC was constructed from a 2 ml syringe (B. Braun Melsungen AG, Melsungen, Germany) and was cooled by a deeply frozen hollow aluminum cylinder at -15 °C. A straw (purchased in a local store) was used to exhale the air through the sampler. The end of the syringe was enclosed with a parafilm wrap to avoid EBC loss. Schematic of the sampler and collection procedure is shown in Fig.1.

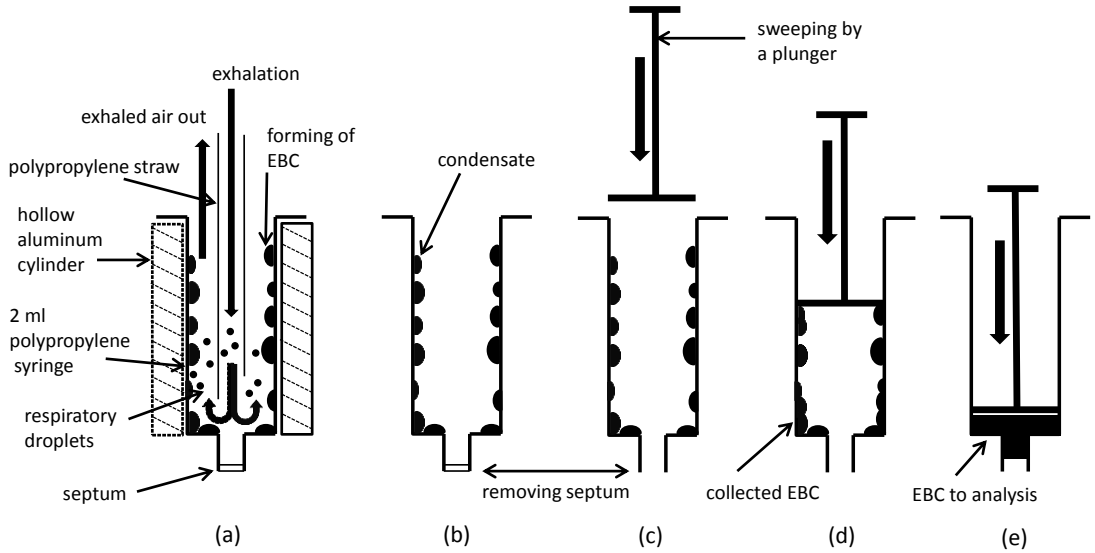

Fig. 1. Schematic of the used miniature EBC sampler (a). An overview of the collection procedure (b)-(e).

3 Results and Discussion

3.1 Analysis of EBC from a single exhalation

Single breath analysis has a significant implication for the future applicability of the EBC as fast and non-invasive diagnostic tool. Using the developed sampler, it is easy to obtain EBC even from individuals that otherwise have problems to breathe over prolonged period (small children, people with serious lung obstruction).

The CE analysis of EBC collected from one individual by repeating the same deep exhalation 5 times is shown in Fig. 2 demonstrating excellent repeatability of the peak areas. This is among other also due to the uniform breathing pattern that is easily kept during a single exhalation.

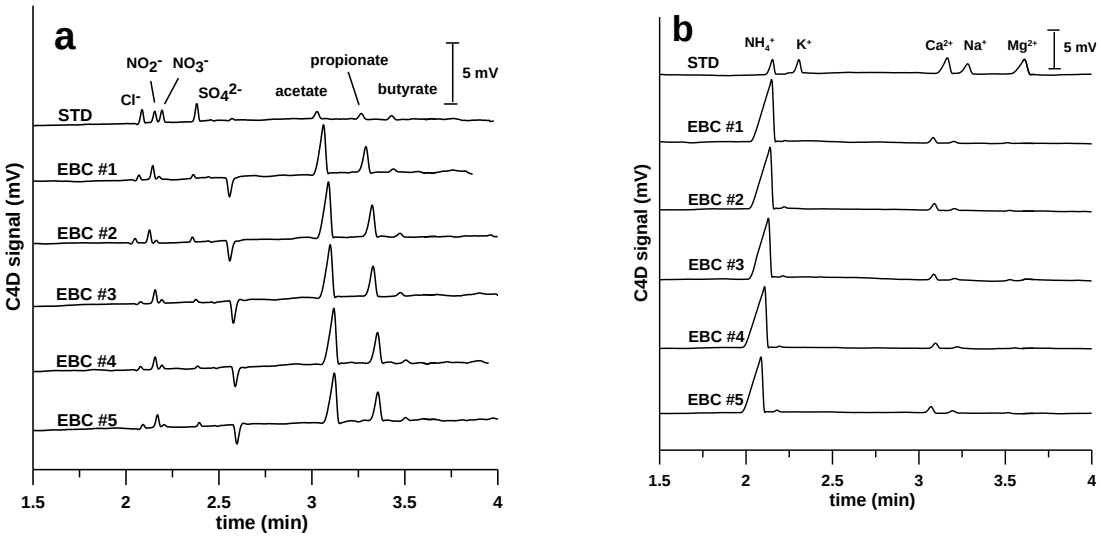

Fig. 2. Electropherograms of a mixture of anions (a) and cations (b). Analysis of 5 samples of EBC obtained from single exhalation.

3.2 Analysis of clinical samples

Figure 3 shows one selected example of clinical diagnosis, in which the anionic trace of a single breath from a healthy volunteer is compared to a person with diagnosed asthma. It has been shown that asthmatic patients have elevated levels of nitrite/nitrate in EBC [2]. In here, we provide a complete anionic scan of the EBC and indeed find out that higher values of NO_2^- was found in the EBC of asthmatic patient compared to a healthy individual. By using the developed sampling method from single breath and CE analysis, the experimental error due to the inadherence to a specific breathing pattern during extended sample collection can be greatly eliminated.

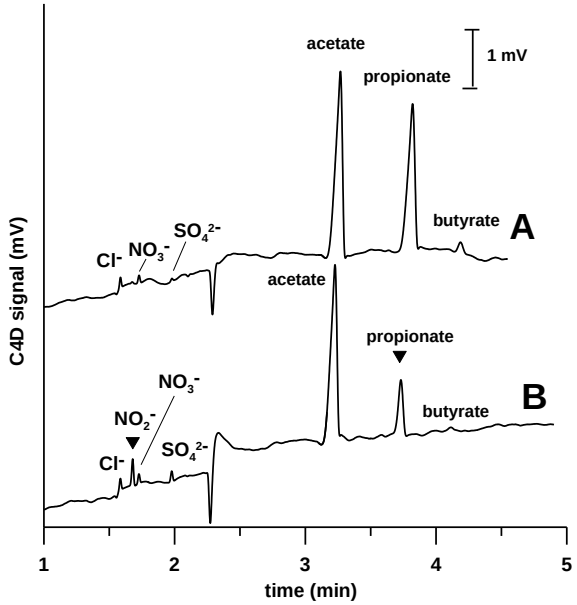

Fig. 3. Comparison of anionic content from a single breath analysis of a healthy person (A) and a person diagnosed with asthma (B).

4 Conclusions

Our laboratory developed miniature sampling device provides a tool for collection of EBC from a single exhalation. The repeatability of the collection procedure was excellent, most probably due to the well controlled ventilatory pattern in this experimental setup. We show practical applicability in the single breath analysis of EBC from both a healthy individual and patient with asthma. The developed methodology may in the future contribute to a wider acceptance of breath diagnosis in clinical practice.

Acknowledgement

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ANALYSIS AND CHARACTERIZATION OF ANTIMICROBIAL PEPTIDES BY CAPILLARY ELECTROPHORESIS

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Summary
Capillary electrophoresis has been applied for quantitative and qualitative analysis, separation and characterization of antimicrobial peptides - halictines. Using this technique, purity degree of synthetic peptides was evaluated, their effective and ionic mobilities and charges within a wide pH range were determined.

1 Introduction

Resistance of bacterial strains to conventional antibiotics has resulted in search for alternative antimicrobial agents. For this purpose, antimicrobial peptides (AMPs) are extensively studied [1]. Among AMPs, halictines – short cationic peptides isolated from the venom of eusocial bee *Halictus sexcinctus* were recently found as new agents with promising therapeutic effects [2]. Their sequence and molecular mass are presented in Table 1. Besides studies on their biological activities, their physicochemical properties have to be determined. Many of them, including charge, size and acid-base properties, can be determined by capillary electrophoresis (CE), which possesses a great potential for analysis and characterization of peptides [3].

Table 1. Halictines sequences and relative molecular masses.

Analog	Sequence	M_r
HAL 1	GMWSKILGHLIR-NH2	1408.8
HAL 1/12	GKWSKILGHLIR-NH2	1405.9
HAL 1/17	KMWSKILGHLIR-NH2	1479.9
HAL 1/19	GKWKKILGHLIR-NH2	1446.9
HAL 1/20	GKWSKILGKLIR-NH2	1396.9
HAL 1/21	GKWKKILGKLIR-NH2	1438.0
HAL 1/27	GMWSKILGHL-NH2	1139.6

2 Experimental

Peptides were synthesized according to the standard N^{α}-Fmoc protocol on a Rink Amide MBHA resin. Crude peptides were purified by preparative RP-HPLC and their relative molecular mass was verified by mass spectrometry.

The CE experiments were carried out in a home made CE apparatus in fused-silica capillary (total/effective length 400/290 mm, id/od 50/375 μm), experimental conditions of peptide analysis are summarized in Table 2. In case of BGE II-VII, hydroxypropylcellulose capillary coating was used, other background electrolytes (BGEs) were used with additive (5% (v/v) polydiallyldimethylammonium chloride).
The analytes were detected by UV-photometric detector at a constant wavelength of 206 nm. Data were collected by Clarity chromatographic station. Purity degrees and effective electrophoretic and ionic mobilities of peptides were calculated by program OriginPro 8.5.0.

Table 2. Experimental conditions of CE analyses of halictine peptides (BGE composition, pH, separation voltage (U) and current (I)).

BGE	pH	Base	Conc. [mM]	Acid	Conc. [mM]	U [kV]	I [μA]
I	2.80	Tris	25.0	H_3PO_4	30.0	-18.0	9.1
II	3.75	Tris	25.0	Form	47.0	17.8	20.0
III	4.25	Tris	25.0	Form	32.0	17.8	20.1
IV	4.75	Tris	25.0	Acet	47.0	17.8	15.5
V	5.25	Tris	25.0	Acet	32.0	17.8	15.1
VI	5.75	NaOH	25.0	MES	75.0	17.8	18.7
VII	6.24	NaOH	25.0	MES	40.0	17.8	20.2
VIII	7.24	NaOH	18.0	H_3PO_4	11.0	-11.8	39.6
IX	7.50	NaOH	17.5	H_3PO_4	10.0	-11.8	31.6
X	7.96	NaOH	17.0	H_3PO_4	9.0	-11.8	43.4
XI	8.93	NaOH	16.7	H_3PO_4	8.4	-11.8	46.2
XII	9.95	NaOH	16.7	H_3PO_4	8.3	-11.8	35.9
XIII	10.93	NaOH	16.7	H_3PO_4	7.6	-11.8	39.5
XIV	11.80	NaOH	18.5	H_3PO_4	4.5	-11.8	40.2

3 Results and Discussion

3.1 Determination of peptide purity

Purity of synthetically prepared peptides was quantified by relative corrected peak area $P_{CA}(i)$, defined by eq. (1) [4], where $A_c(i)$ is the corrected area of the *i*-th peak (peak area divided by its migration time). Peptide purity was found to be in the range 97-100%. As shown in Figure 1A., two impurities were found in the analysis of peptide HAL 1/17, despite 100% purity degree of this peptide was found by RP-HPLC. On the other hand, 100% chromatographic purity degree was confirmed by CE analysis of peptide HAL 1 as demonstrated in the Figure 1B.

$$\boldsymbol{P_{CA}(i)} = \frac{\boldsymbol{A_c(i)}}{\sum \boldsymbol{A_c(i)}} \qquad (1)$$

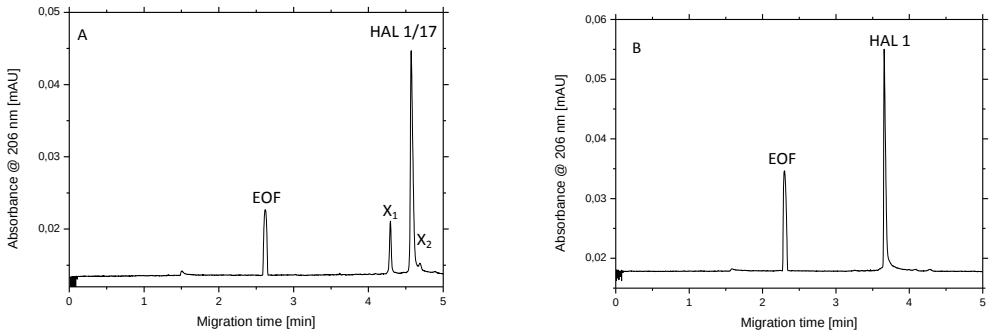

Fig. 1. CE analysis of peptides HAL 1/17 (A) and HAL 1 (B) in BGE I. For sequences of peptides see Table 1 and for CE experimental conditions see Table 2.

3.2 Separation of peptide mixtures

Because of a large number of tested peptides, we decided to analyze mixtures of halictines to decrease the total analysis time. Separation of peptides is strongly dependent on the differences of their specific charge, therefore the peptide specific charges were calculated as a function of pH.

3.3 Determination of effective mobilities

Key parameter in CE is the electrophoretic mobility, which can be used for calculation of physicochemical characteristics. Figure 2 shows effective mobilities of peptides as a function of pH. Mobilities were calculated from experimental data according to eq. (2) and (3) [5]:

$$m_{eff} = \sum_{i=1}^{n} m_i x_i \qquad (2)$$

where m_i is ionic mobility and x_i is molar fraction of the *i*-th species defined as:

$$x_i = c_i / \sum_{i=1}^{i=n} c_i \qquad (3)$$

where c_i is the molar concentration of the *i*-th species.

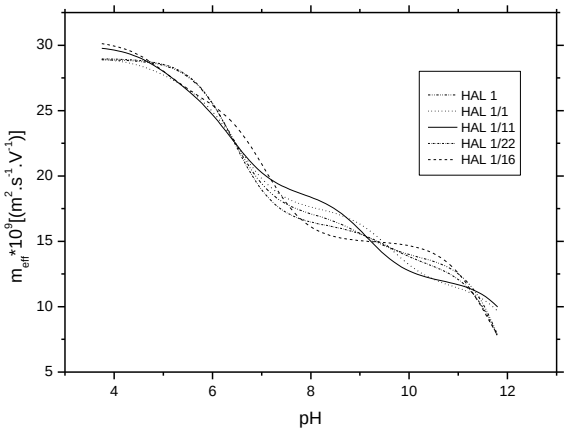

Fig. 2. Calculated effective mobilities (m_{eff}) of peptides as a function of pH.

4 Conclusions

In addition to purity analysis of synthetic antimicrobial peptides, capillary electrophoresis proved to be an effective method to characterize their physicochemical properties with advantages such as low analyte and electrolyte consumption and short time of analysis.

Acknowledgement

The work was supported by the Czech Science Foundation (projects nos. P206/12/0453, 13-17224S) and by the ASCR (Research Project RVO 61388963).

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TAIL-LABELED OLIGONUCLEOTIDE PROBES FOR A DUAL ELECTROCHEMICAL MAGNETIC IMMUNOPRECIPITATION ASSAY OF DNA-PROTEIN BINDING

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Summary

A novel assay for detection of DNA-protein binding has been developed. Oligonucleotides bearing or lacking specific binding site of the p53 protein were tail-labeled by two different modified deoxynucleotide triphosphates using terminal deoxynucleotidyl transferase. Electrochemical detection enabled to discriminate between sequence-specific and non-specific p53-DNA binding in a competition assay.

1 Introduction

Creating 3´OH tails by terminal deoxynucleotidyl transferase (TdT) can be used for labeling DNA. TdT attaches nucleotides at the 3´OH terminus of DNA using dNTPs as substrates [1]. Previous data show that DNA tail-labeled by TdT is convenient for immunoprecipitation binding experiments with tumor suppressor protein p53 and that

sequence-specific and non-specific DNA binding can be distinguished [2]. However, for competition assays (enabling a direct determining which DNA probe is chosen by the protein from a mixture) utilization of a single DNA label is insufficient. Therefore a new approach based on labeling by two different modified deoxynucleotide triphosphates is presented.

2 Experimental

2.1 Tail-labeling of oligonucleotides

dATPs modified with either nitrophenyl or benzofurazan were used for tail-labeling of two types of single-stranded oligonucleotides (containing or lacking p53 binding site; PGM1 or noCON) by TdT. The reaction mixture contained 3.6 μM ss oligonuleotide and 1.8 mM modified dATP; the reaction was conducted at 37 °C overnight.

2.2 p53-DNA binding

A previously introduced magnetic beads-based immunoprecipitation assay [3] was used for the protein-DNA binding. The p53 protein was mixed with Bp53-10.1 antibody and incubated for 20 minutes on ice. Then the tail-labeled duplexes at p53 tetramer/duplexes ratio 3:1 were added and incubated 30 minutes on ice. Reaction mixture was added to washed magnetic beads covered with protein G and incubated for 30 min at 10 °C. Oligonucleotides were released from the magnetic beads-antibody-protein-DNA complex through a 5-min incubation at 65 °C in 10 μl of 0.5 M NaCl.

2.3 Electrochemical analysis

Cyclic voltammetry at a hanging mercury drop electrode (HMDE) was used for analysis of the released tail-labeled probes. DNA was accumulated at the electrode surface from 4 μl aliquots for 60 s. CV settings were: initial potential 0.0 V, switching potential -1.85 V, final potential 0.0 V, scan rate 1 $V.s^{-1}$, step potential 5 mV.

3 Results and Discussion

In addition to two peaks typical for DNA (an anodic G peak at potential -0.25 V and a cathodic CA peak at -1.5 V), a cathodic peak typical for NO_2 reduction at -0.5 V [2] for oligonucleotides tail-labeled with $dA^{NO2}TP$ or a cathodic peak typical for benzofurazan reduction at -0.9 V [4] for oligonucleotides tail-labeled with $dA^{BF}TP$ could be observed in a cyclic voltammogram of DNA probes released from the magnetic beads. When the labeled oligonucleotides were used for the binding experiments, respective peaks were well developed. Results gained for the competition binding experiment show a decrease in the intensity of the peaks since the labeled DNA probes mutually competed for the protein. Notably, only a slight decrease was observed for the peak gained for the PGM1 probe (e.g. peak BF, where PGM1 probe was labeled with BF and noCON with NO_2) while the signal of the

noCON probe decreased more significantly. This behavior corresponds to the known binding specificity of the p53 protein.

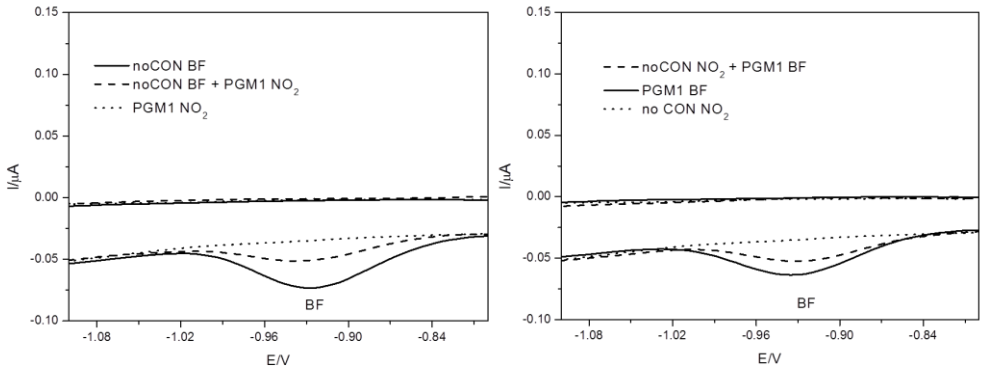

Fig. 1. Sections of voltammograms obtained for oligonucleotides PGM1 and noCON tail-labeled with benzofurazan. a) p53-DNA binding experiment with noCON probe labeled with benzofurazan compared with competition binding experiment with BF-noCON and NO_2-PGM1. b) p53-DNA binding experiment with PGM1 probe labeled with benzofurazan compared with competition binding experiment with BF-PGM1 and NO_2-noCON.

4 Conclusions

We present a new methodology which enables to discriminate between sequence-specific and non-specific p53-DNA binding when using $dA^{NO2}TP$ and $dA^{BF}TP$ tail-labeled oligonucleotides as DNA probes. However, in order to determine exact level of sequence-specific and non-specific p53-DNA binding and to allow quantification of the gained results, more experiments need to be conducted.

Acknowledgement

This work was supported by the Czech Science Foundation (grants P206/12/G151 and 204/07/P476) and by the ASCR (RVO 68081707).

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MAGNETIC BEAD-BASED IMMUNOCAPTURE OF CLINICAL BIOMARKERS IN MICROFLUIDIC DEVICES: FROM PEPTIDES TO WHOLE CELLS

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Summary
Detection of specific clinical biomarkers occurring mostly in low concentrations in body fluids is highly challenging, therefore special techniques for their isolation and preconcentration from complex biological samples is required. Immunocapture of two diverse biomarkers such as peptides (amyloid β) and/or cells (circulating tumour cells) using magnetic immunosorbent (MIS) was performed inside two microfluidic devices tailored specially for each biomarker. Results achieved on development of MISs for microfluidic purposes as well as its evaluation inside the microfluidic devices in past 6 years are presented in here.

1 Introduction

Isolation and detection of specific biomarkers of serious diseases, such as Alzheimer´s disease (AD) and/or adenocarcinomas, is still challenging task to solve. In our research the well-established technique, the immunoaffinity separation on magnetic carrier, was implemented into a microfluidic devices. For isolation of two diverse biomarkers, in case of Alzheimer´s disease it was 4 kDa peptide amyloid β (Aβ) while in case of adenocarcinomas it was rare blood cells called "circulating tumour cells" (CTCs), the magnetic immunosorbents (MIS; magnetic microparticles coated with specific antibody against the biomarker) were applied. The process of particle selection, the antibody (Ab) immobilization technique consideration and the preparedMIS evaluation was performed. Subsequently, each MIS was integrated into specifically developed microfluidic polydimethylsiloxan (PDMS) device (Institut Curie, Paris, F) inside which the MIS behavior was observed. The microfluidic device for Aβ peptides preconcentration forms the MIS into a plug inside the straight channel by the action of two strong rare-earth permanent magnets [1]. For circulating tumour cells expressing epithelial cell adhesion molecule (EpCAM), sophisticated diamond-tree-shaped device called Ephesia chip was employed [2]. Such microfluidic system has two large capture chambers with magnetic pattern to guide the MIS where the microcolumns should be created in the presence of homogenous magnetic field coming from magnetic coil surrounding the device.

2 Experimental

For anti-Aβ MIS, 1μm silica magnetic particles SiMAG-active (Chemicell, Berlin, D) with eight various functional groups covalently coated with monoclonal IgG1 (mAb)

to Aβ (1-16 AA, clone 6E10) from Covance (Princeton, NJ) or rabbit polyclonal IgG (pAb) to Aβ (1-14 AA) from Abcam (Cambridge, UK) were applied [3, 4]. Such MIS was used in microfluidic device illustrated in Fig. 1.

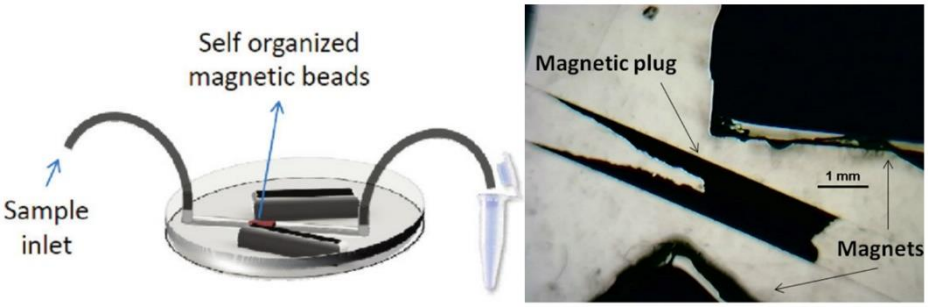

Fig. 1. Simple straight channel PDMS device with one inlet/outlet and two magnets arranged in 20° angel towards the channel trapping magnetic particles into a plug [1].

For anti-EpCAM MIS, monodisperse (4 μm) macroporous cross-linked poly(glycidyl methacrylate) (PGMA) microspheres coated with human serum albumin (HSA) and/or poly(ethylen glycol) (PEG) with covalently immobilized mouse monoclonal anti-EpCAM antibody IgG1 (HEA 125) provided by PROGEN Biotechnik (Heidelberg, D), were employed [5, 6]. The MIS was implemented inside the Ephesia chip (Fig. 2).

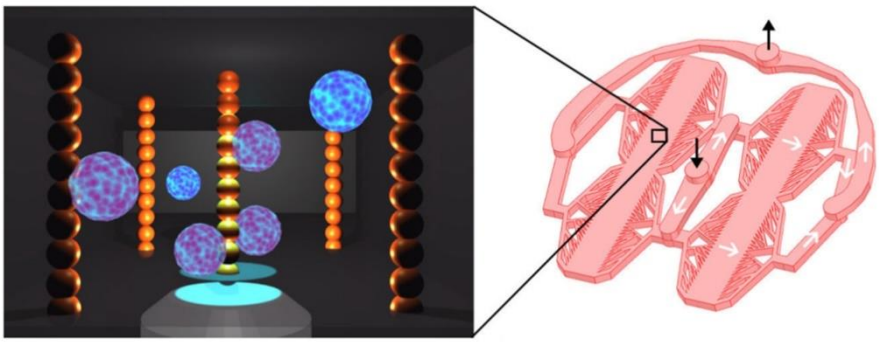

Fig. 2. Ephesia chip with diamond-tree like architecture with two immunocapture chambers with microcolumns made of MIS with anti-EpCAM specificity [2].

3 Results and Discussion

3.1 Antibody clone selection

During this research, technique for comparison of various monoclonal antibody clones directed against the target epitope was developed. Conventional dot-ELISA technique was improved as we enriched it with a chaotropic reagent step. We named the technique Dot-ELISA Affinity test. The method enabled us to predict which monoclonal antibody clone has appropriate affinity to our target (Aβ or EpCAM in case of CTCs). This property is crucial to know when efficient MIS should be developed.

3.2 Magnetic immunosorbent for on-chip immunoprecipitation of Aβ peptides

Magnetic particles with covalently immobilized anti-Aβ IgG was evaluated firstly in batch arrangement in Eppendorf tube using magnetic separator. The isolated peptides were analysed by urea-Tris-Tricine-SDS-PAGE or by MALDI-TOF-MS. When satisfactory results were achieved, on-chip immunoprecipitation, called micro-immunoprecipitation (μIP), was performed. Using the μIP, we were able to preconcentrate the Aβ peptide from the volume 100 μl to 0.58 μl (approx. 170 fold) and moreover to obtain peptide in high purity presented in reagent suitable for subsequent separation and/or detection method. Thus, μIP was found as efficient method appropriate for Aβ peptides preconcentration in micro-volumes. Moreover, further experiments showed possible connection with microchip capillary electrophoresis that was performed even with AD cerebrospinal fluid samples [7].

3.3 Magnetic immunosorbent for on-chip immunocapture of EpCAM+ cells

PGMA particles coated with HSA or PEG were used for covalent immobilization of anti-EpCAM IgG. The prepared MISs were evaluated in batch arrangement for their immunocapture efficiency of EpCAM-expressing cells. Four cell lines with various EpCAM density were employed (MCF7+++, SKBR3++, A549+, Raji-). These experiments were compared with commercial MIS and similar results were achieved. Finally, evaluation in a microfluidic Ephesia chip was demonstrated using the MCF7 cell line, a model system for CTCs.

4 Conclusions

The magnetic bead-based immunocapture technique provide many benefits, such as mild conditions for the biomarker, easy purification enabling downstream processing and/or isolation from complex biological samples (cerebrospinal fluid, serum or cell mixture). These are supported by advantages offered by microfluidic approach, low-sample/reagent consumption, high throughput and/or integration. This was performed on two diverse clinical biomarkers, Aβ and CTCs, analysed inside microfluidic devices designed for such specific approaches.

Acknowledgement

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DIODE LASER THERMAL VAPORIZATION – NOVEL SAMPLE INTRODUCTION TECHNIQUE FOR ICP MS

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Summary
Diode laser thermal vaporization (DLTV) is a novel technique of sample introduction to inductively coupled plasma mass spectrometry (ICP MS). A continuous infrared diode laser is used to generate dry aerosol by pyrolysis of a suitable substrate (paper, cellulose) carrying a dried sample. DLTV ICP MS presents a rapid and reproducible (RSD ~10%) analysis of many common metals, such as Co, Ni, Zn, Mo, Cd, Sn, Pb etc. with limits of detection in low pg range [1]. Furthermore, DLTV ICP MS can be combined with the TLC separation for simple elemental speciation. The DLTV approach was demonstrated on the determination of lead and cadmium in whole blood [2] and on cobalamin (CA) speciation in dietary vitamin supplement tablets [3].

1 Introduction
ICP MS is a versatile tool for trace elemental analysis. Introduction of liquid samples is typically carried out using pneumatic nebulizer systems, because of their simplicity and low cost. Low sample transport efficiency and high sample consumption of the

nebulizers promoted the development of new non-nebulizing sample introduction techniques, such as electrothermal vaporization (ETV) [4] and substrate-assisted laser desorption (SALD) [5-7]. The presented sample introduction technique, DLTV, unlike SALD or LA, uses a NIR diode laser rather than an expensive high-energy pulse laser. To facilitate substrate pyrolysis, the black ink absorber is printed onto the substrate. A submicroliter liquid sample is deposited on the substrate, dried, vaporized in the laser ablation chamber and the generated aerosol is carried into the ICP MS. Advantages of this technique are low cost, minimal sample treatment and consumption, optional use of prearranged calibration sets, possibility of sample archiving and transportation.

2 Experimental

2.1 Sample preparation

Black ink (HP CB316E, Hewlett Packard, Ireland) rectangles (2.5 × 1.0 mm) or lines for TLC (1.0 × 8.0 mm) were printed over the substrate using a commercial inkjet printer (HP Photosmart C5380). The Pb and Cd standard solutions (Analytica, Czech Republic) and human blood standard (BCR-634) were deposited on the preprinted rectangles as 200 nL droplets using a micropipette or by a piezoelectric dispenser MJ-ABP-01 (MicroFab Technologies, Texas, USA). For TLC of CAs, the volume of 0.5 μL of the sample was spotted onto the TLC start by micropipette.

2.2 Thin-layer chromatography

Aluminum-backed TLC cellulose plates (HX267159, Merck, Germany) spotted with CA standards (10 $\mu g.L^{-1}$ – 4 $g.L^{-1}$) and water extract of vitamin tablets were eluted in the dark with a mixture of 1-butanol : ethanol : water = 10 : 3 : 7 (v/v/v) containing 0.5 % (v/v) of concentrated NH_3 solution for two hours.

2.3 Laboratory-built device for DLTV

Glass tube (i.d. 3.8 mm, o.d. 6 mm, length 170 mm) was used as a vaporization cell. A continuous-wave 1.2 W, 808-nm diode laser module (RLDH808-1200-5, Roithner LaserTechnik, Austria) was attached to the slider of a syringe pump, which provided the translation for the line scan of the laser beam along the substrate strip (Fig. 1).

3 Results and Discussion

3.1 Determination of Pb and Cd in dried blood

Lead and cadmium were determined in the human blood certified reference material BCR-634, which contains 46 ± 5 $mg.L^{-1}$ of Pb and 1.4 ± 0.4 mg L^{-1} of Cd, using the multiple standard addition technique. A prearranged multi-elemental calibration set was constructed with the piezoelectric dispenser and the 200 nL blood droplets were deposited on the prepared positions. The ion signal of ^{208}Pb and ^{111}Cd was recorded; the trace for ^{208}Pb is shown in Fig. 2A. Determined contents were 47 ± 4 $mg.L^{-1}$ for Pb and 1.7 ± 0.6 $mg.L^{-1}$ for Cd, in perfect agreement with the certified values.

3.2 TLC - DLTV ICP MS

TLC - DLTV ICP MS performance was evaluated from the developed TLC chromatograms of dilution series from 1 $g.L^{-1}$ down to the concentration of 10 $\mu g.L^{-1}$ for each CAs (Fig.2B). As expected, DLTV allows detection down to 10 $\mu g.L^{-1}$ (LOD ~2 pg for each CA), five orders of magnitude below visual detectability. Single specie identified in the vitamin supplement was cyanocobalamin (CN-CA). The determined content of CN-CA was 130 ± 12 µg per tablet using DLTV ICP MS. This value was confirmed by the traditional solution analysis of tablet extract using nebulizer ICP MS.

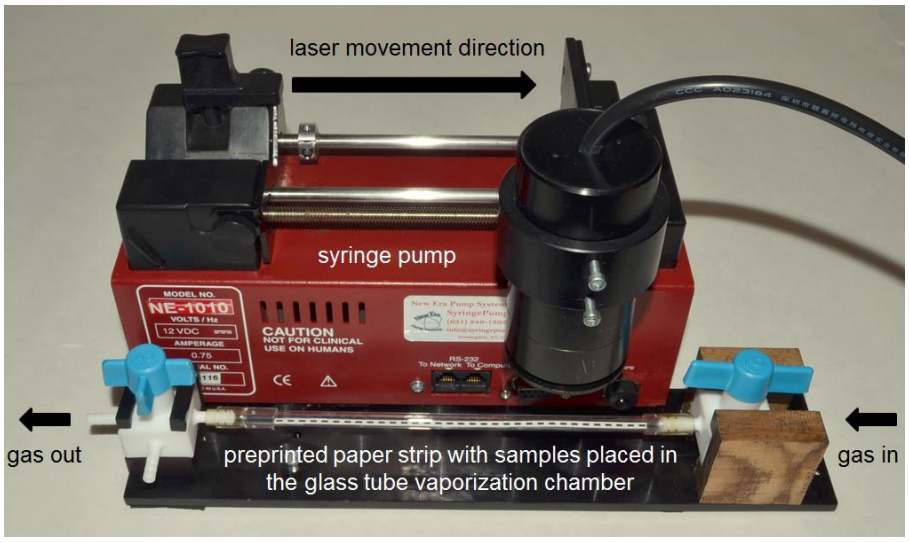

Fig. 1. Laboratory-built device for DLTV.

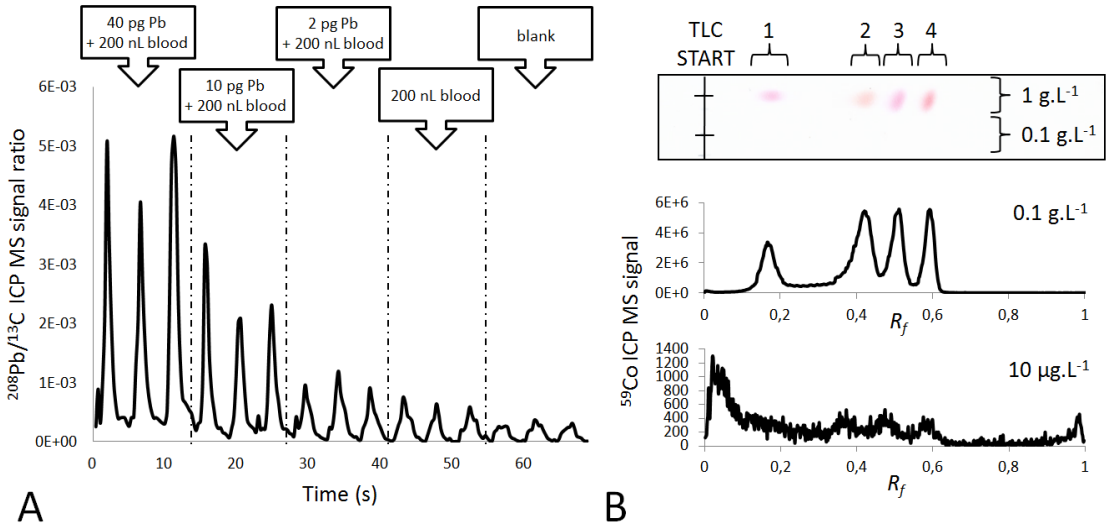

Fig. 2. A) Time record of the ICP MS signal in a DLTV line scan along the paper strip for determination of Pb in whole blood. B) Visual and DLTV ICP MS detection of developed TLC plate. 1, hydroxocobalamin; 2, adenosylcobalamin; 3, cyanocobalamin; 4, methylcobalamin.

4 Conclusions

A system for sample introduction to inductively coupled plasma, DLTV has been developed. The whole blood reference material was simply deposited without any pretreatment on the prearranged standard calibration set and the content of Pb and Cd was determined by DLTV ICP MS using the simple laboratory built device. The determined contents of Pb and Cd, 47 ± 4 $mg.L^{-1}$ and 1.7 ± 0.6 $mg.L^{-1}$, respectively, matched precisely with the certified reference values. Furthermore, feasibility of DLTV ICP MS as a detection tool for TLC was examined on quantitative analysis of CAs in the vitamin supplement. CN-CA with content 130 ± 12 μg per tablet was the only specie found in the vitamin supplement.

Acknowledgement

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A RAPID IDENTIFICATION OF TRIACYLGLICEROLS AND PHOSPHOLIPIDS USING MALDI-TOF MS

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Summary
High performance liquid chromatography coupled with mass spectrometry is widely used separation technique in analysis and identification of phospholipids. Aim of the study was the separation, identification and comparison of the composition of phospholipids and triacylglycerols extracted from biological matrices using MALDI-TOF MS. By using solid phase extraction was separated polar lipids from the non-polar lipids. Additionally, compare home-made column in separation of phospholipids from various samples.

1 Introduction

Research has shown that phospholipids (PL) play a variety of biological functions and are fundamental to many life processes. Special role stems from the fact that, along with cholesterol and proteins, they are an important component of biological membranes deciding on their semi-permeable nature [1]. Due to their amphiphilic structure PL mediate the transport of many of the compounds across biological membranes, very often form complexes with proteins or lipoproteins (e.g. plasma). Phospholipids play a crucial role in the inhibition of degenerative liver, heart, blood vessels and nervous system diseases [2]. Choline included in lecithin is essential for the synthesis of the neurotransmitter acetylcholine, regulating inter alia breathing, heart function and processes associated with remembering and concentrating [3]. In addition, a protective role in relation to the prevention of liver cells accumulation of fat in them [3,4]. Phospholipids can be analyzed by thin layer chromatography (TLC) [5], high performance liquid chromatography (HPLC) [6], gas chromatography (GC) [7]. In order to identify the PL used mass spectrometry, especially electronspray ionization (ESI), fast atom bombardment (FAB) and matrix-assisted laser desorption/ionization (MALDI) [8].

2 Experimental

2.1 Chemicals

Methanol, chloroform, sodium chloride and SPE cartridges employed for the extraction procedure were obtained from Sigma-Aldrich (Steinheim, Germany). Methanol, acetonitrile and formic acid for HPLC analyses were purchased from Sigma-Aldrich (Steinheim, Germany). The standard phospholipids were obtained from Larodan Lipids (Malmö, Sweden). MALDI matrix DHB and HCCA was

obtained from Fluka (Neu-Ulm, Germany). Ground steel targets (Bruker Daltonik, Bremen, Germany) were used for sample deposition.

2.2 Lipids extraction

The extraction of the lipid fraction of milk and egg samples was carried out according to the modified Folch et al. procedure [9].

2.3 Solid phase extraction SPE

Lipids extracts (50 mg) were dissolved in 1 mL of chloroform/methanol (95:5, v/v) mixture and cleaned using SPE cartridge (500 mg of silica gel). The SPE cartridges were first conditioned with 5 mL of chloroform/methanol (95:5, v:v) mixture. Neutral lipids were eluted with 10 mL of chloroform/methanol (95:5, v:v) mixture. Phospholipids were eluted with 5 mL of methanol and 5 mL of chloroform/methanol/water (3:5:2, v/v/v) mixture. The solutions were evaporated at 37°C and re-dissolved in 1 mL of chloroform/methanol (2:1, v/v) mixture for LC analysis.

2.4 Chromatographic system

Separation of lipid classes was accomplished in a Shimadzu Prominence HPLC system (Tokyo, Japan). Instrument control, data acquisition and processing were performed with LabSolution software for HPLC. A 125 × 4.6 mm home-made silica and N,O-dialkylphosphordiamidate column (C12) with d_p=5 µm particle diameter and 100 Å pore diameter was used. The injection volume was 1 µL. Analyses were performed at 30°C. Table 1 show the chromatographic condition.

Table 1. Chromatographic condition of mobile phase.

Type of stationary phase	Mobile phase
Silica	ACN : H_2O (0 min 100% ACN; 5 min 90% ACN; 15 min 80% ANC; 35 min 50% ACN)
Di-Amino-PC12	80% MeOH : 20% H_2O

2.5 MALDI system

Mass spectrometric measurements were performed using a MALDI-TOF/TOF MS instrument (Bruker Daltonics, Bremen, Germany). All spectra were acquired in reflector positive and negative mode within a *m/z* range of 200–1600. Fragment spectra were determined using the LIFT post source decay (PSD) technique over a *m/z* range of 50–1000.

3 Results and Discussion

PL classes were analyzed using home made column (silica, Di-Amino-PC12). The separation mechanism based on the different of polarity “headgroup” phospholipids. Each phospholipids fraction was collected manually and identified by MALDI-TOF MS. Figure 1a shows the mass spectrum of the phospholipids in the *m/z* 670-1000.

The phosphatidylcholine (PC) class of phospholipids is the dominant in the milk samples. For the samples of milk the dominant are monounsaturated fatty acids (MUFA). Fig. 1b presented mass spectrum of TAGs *m/z* 670-1000. The fatty acids in the separated fraction range from C50:2 to C52:4.

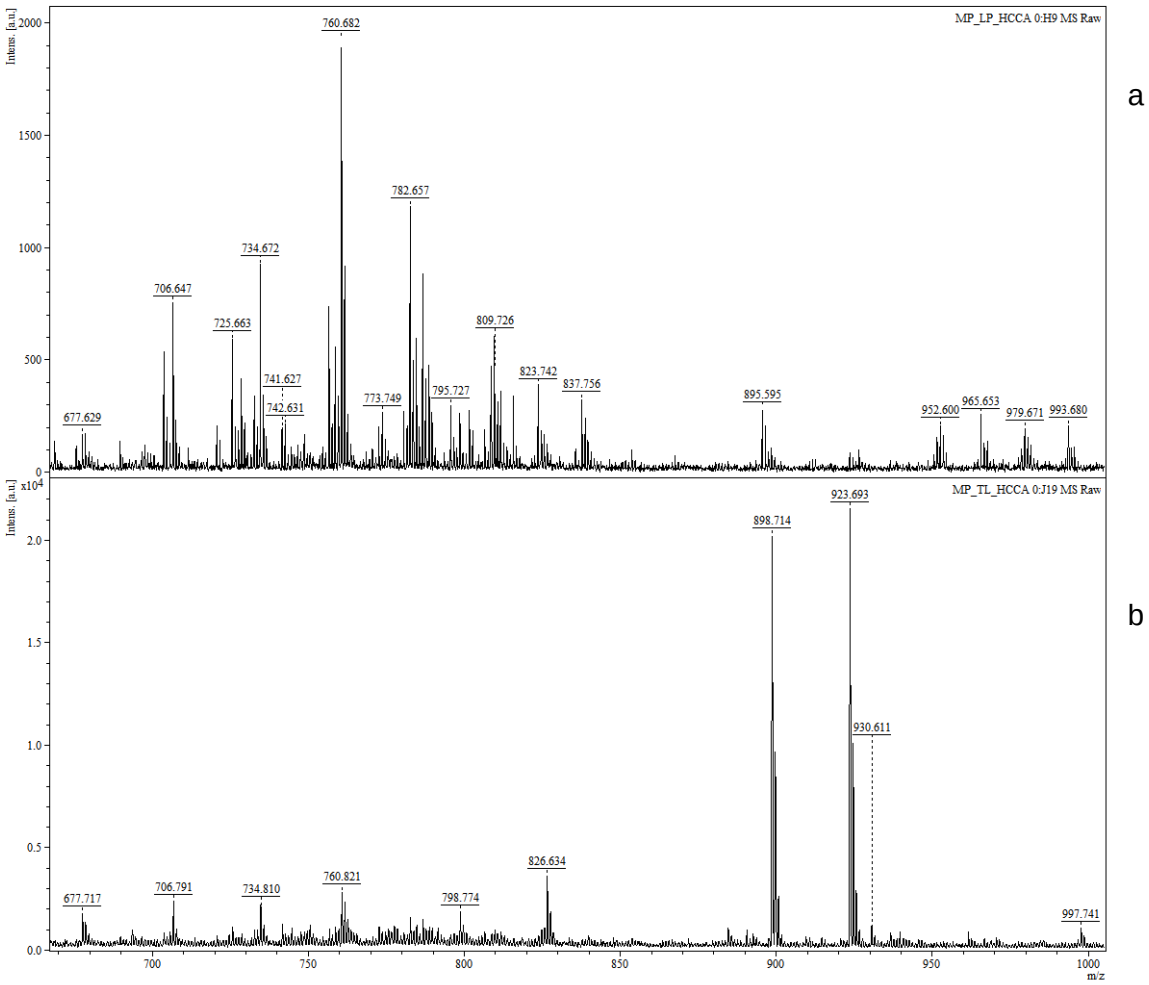

Fig. 1. Partial (m/z 670–1000) MALDI-TOF mass spectra of milk phospholipids (a), and TAGs (b) fraction collected from the SPE cartridge.

4 Conclusions

Analysis of a sample including phospholipid and TAG components by MALDI-TOF MS resulted in mass spectra dominated by the phospholipids. SPE separation, mixture resolution was sufficient to allow detection of both TAGs and phospholipids from a complex mixture. New stationary phase (Di-Amino-PC12) exhibits good selectivity in analysis of phospholipids, according to polar head in reserved phase conditions.

Acknowledgement

This study was supported by grants from European Social Found, the Polish National Budget and the budget of the Kujawsko-Pomorskie Region as a part of the "Krok w przyszłość V" (Step into the Future) and grant from Ministry of Science and Higher Education, Grant no. NCN 2013/11/N/ST4/01838 for period 2014–2016.

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COMPARISON OF CHIRAL STATIONARY PHASES BASED ON CYCLOFRUCTAN IN NORMAL PHASE LIQUID CHROMATOGRAPHY

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Summary

Cyclofructans belong to the newest type of chiral selectors. In this study two derivatized-cyclofructan based chiral columns were used for the enantiomeric separation of the set of new biological active racemic compounds using liquid chromatography under normal phase conditions and obtained results were compared.

1 Introduction

Cyclofructans (CFs) are the new type of chiral stationary phases (CSPs) developed in the last few years. Derivatized cyclofructans, with aliphatic or aromatic functionalities, show improved separation abilities in analysis of a wide range of chiral analytes. Enantiomers separation using cyclofructan-based chiral stationary phases can be carried out under normal phase, reversed-phase and polar organic separation modes. One advantage for all cyclofructan-based chiral stationary phases is their excellent stability in all common organic solvents and more aggressive acidic and basic additives [1, 2].

Phytoalexins represent the group of low molecular weight secondary metabolites synthesized by plants. These compounds as well as their newly synthesized chiral derivatives possess great attractivity nowadays due to their antibacterial, antifungal and chemoprotective properties [3].

In the present study, the separation performance of two derivatized-cyclofructan based CSPs, RN-CF6 and IP-CF6, was examined by separating the set of the new biological active racemic analytes under normal phase mode.

2 Experimental

The set of seven new racemic derivatives of spiroindoline phytoalexins used as test analytes were synthesized at the Department of Organic Chemistry (P. J. Šafárik University, Košice). Two derivatized-cyclofructan chiral columns based on R-naphthylethyl carbamate cyclofructan 6 (RN-CF6) and isopropyl carbamate cyclofructan 6 (IP-CF6) were tested.

3 Results and Discussion

In the normal phase elution mode, the mobile phases composed of n-hexane and 2-propanol or ethanol as polar modifiers in various rations were used for the enantioseparation of studied compounds. The influence of the mobile phase composition, type and concentration of the polar modifier as well as the mobile phase additives on the chromatographic parameters were investigated. The results obtained for two different chiral stationary phases were compared (Fig. 1.). The effect of column temperature on the separation of enantiomers was also studied.

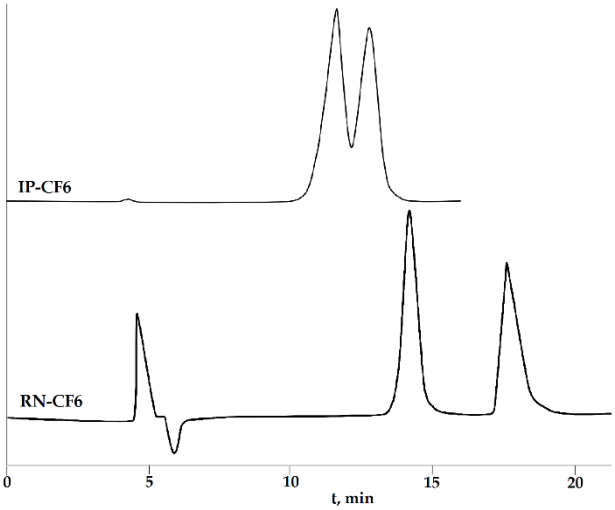

Fig. 1. Chiral separation of racemic analyte on two different CSPs. Mobile phase composition: n-hexane/2-propanol (90/10 v/v); temperature 25°C; detection 250 nm.

4 Conclusions

The separation performance of two derivatized-cyclofructan chiral stationary phases was compared under normal phase mode. The set of new biologically active racemic

analytes were used for the evaluation of results. It was shown that the type of chiral selector, mobile phase composition as well as the structure of the test analytes affect the chiral separation.

Acknowledgement
This work was supported by the internal grant VVGS-PF-2014-448 and the Scientific Grant Agency of the Slovak Republic VEGA (No. 1/1096/12).

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SIMULATION OF MICROFLUIDIC SYSTEMS WITH COMSOL MULTIPHYSICS

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Summary
In microfluidic devices, especially if those include complicated channel system, alterations between the experimentally gained and the expected (desired) flow rates at given parts in the microchip are often obtained. Without the use of simulation softwares it is quite difficult to get to the right channel pattern or the best experimental parameters (flow rates, pumping pressure). In our work the applicability of COMSOL Multiphysics [1] is studied for special chip designs.

1 Introduction
COMSOL Multiphysics is a Finite Element Method (FEM) based simulation software for various engineering applications. The Computational Fluid Dynamics (CFD) module of the program is expected to be well-applicable for microfluidic chips, too. This software was used for the simulation of the velocity field in a channel system,

which included many junctions of channels and bottlenecks (microchip with injectors and 12 chromatographic packings [2]).

2 Experimental

Previously we have already reported on a new packing procedure of chromatographic particles, where we created a bottleneck in a channel of 100 micron width. Around 1 microliter of suspension of methanolic C18 particles was injected into the chip channel toward the bottleneck and then washed with methanol [3]. The particles retained around the bottleneck, and then the newly arrived particles adhere smoothly to the packing; increasing its length continuously. The first particles acted as keystones blocking the other particles [4]. With the use of this procedure parallel chromatographic packings were created in the system and the separation of a food dye mixture was performed (Fig.1).

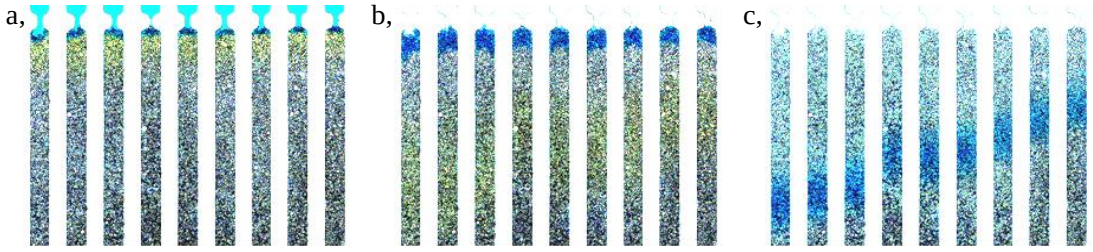

Fig. 1. A single sample (V: 0.5 µL) was injected to the chip and it was splitted to several equal parts before the packings (a,). While the blue dye completely retained at the beginning of the packing, the yellow dye was washed out by water mobile phase (b,). Changing the mobile phase to methanol the blue dye was eluted (c,).

According to our experimental results the flow rate in a system containing twelve parallel channels continuously decreased and the ratio of the maximal and minimal velocity in the parallel channels was around 2.5 [2].

3 Results and Discussion

A continuous diminish of the flow rates can be seen in the pressure dispersion (Fig. 2a) and velocity magnitude (Fig. 2b) diagrams, the velocity in the first channel is almost 2.5 times higher than in the last channel. That is the COMSOL simulations matched well with the experimental results.

We made simulations for the equalization of the velocities in the parallel channels. We studied the effect of opening or closing the certain ends of the channels, application of pressure or vacuum at several parts of the chip, but the best result was achieved with the modification of the channel pattern. With the modification we obtained a 1.05 ratio for the maximum and minimum velocities (Fig. 3).

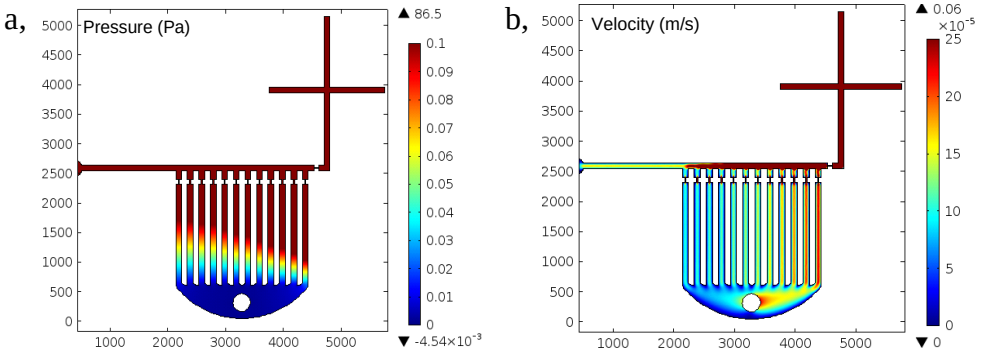

Fig. 2. Results of COMSOL simulations. Diagrams of pressure dispersion (a) and velocity magnitude (b) in microchip.

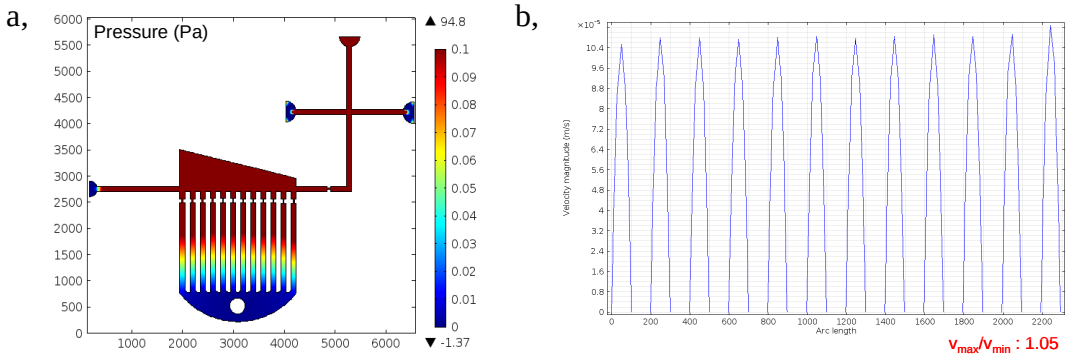

Fig. 3. Equalization of the velocities by the modification of the channel pattern. Pressure dispersion (a), and linear velocity (b) diagrams (which is obtained after cutting the channels with a line and measuring the velocity in each channel).

4 Conclusions

The COMSOL Multiphysics was successfully applied for the simulation of microfluidic systems. Using this simulation software the exhausting preparation of microfluidic chip (including lithographic masks, molds) with trial and error method can be minimized.

Acknowledgement

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INFLUENCE OF MASS SPECTROMETRY RESOLUTION ON METABOLITE COVERAGE IN PLASMA

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Summary
Finding of optimal resolution for metabolite rich biofluids was done by *in silico* calculations and analyzes of human plasma by LC-HRMS. Number of dedicated metabolite increased with increasing resolution up to 120k. Which is necessary for metabolomic analysis.

1 Introduction

High resolution accurate mass MS and MS^n is useful tool in metabolite identification, but has its limitations. The aim of this work is to find what resolution should be sufficient for metabolomics analyses and determine number of possible overlapping compounds. This was addressed by *in silico* calculations and analyzing human plasma by LC-HRMS at different resolutions.

2 Experimental

In silico calculations based on HMDB database (http://www.hmdb.ca/) containing 41 448 metabolites were run in R software to see which groups of metabolites are experiencing most of these "overlaps". Analyses of the same sample can deliver different number of compounds when using various resolutions. R package Rdisop was used to calculate isotopic patterns and monoisotopic masses from chemical formula of each compound. Afterwards the common adducts were added to the list ($[M+NH_4]^+$, $[M+K]^+$, $[M+Na]^+$, $[M+ACN+H]^+$, $[M+Na-2H]^-$, $[M+Cl]^-$, $[M+CH_3COO]^-$, $[M+K-2H]^-$, $[M+FA-H]^-$). These models were calculated for increasing resolutions from 15k up to 3840k in both – positive and negative mode.

To evaluate these models LC-MS of one identical plasma sample were run on Orbitrap Elite (Thermo Fisher Scientific, CA, USA) from 15k up to maximum resolution of the instrument 480k, with simple separation conditions. The XCMS package in R and Thermo Excalibur 2.2 SP1.48 (Thermo Fisher Scientific, CA, USA) were used for data evaluation.

3 Results and discussion

In silico calculations based on database determined 15 711 unique masses without isobars (m/z range 70 - 2000 Da). Number of distinguishable masses was calculated for up to 3840k resolution. *In silico,* the number of unique masses plateaued at the inflection point of 240k resolution.

In real plasma samples this plateau was observed at 60k – 120k resolutions in LCMS experiment, in both positive and negative. This should be further tested to exclude possible "processing artifacts". The maximum number of compounds observed in positive mode in mass range 70 – 2000 m/z was 4100 peaks.

4 Conclusions

Results suggest that resolutions of >120k are needed for analyses in metabolite rich biofluids. Together with simple separation method we can achieved high throughput workflow for metabolite identification.

Acknowledgement

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STUDY ON SILVER IMMOBILIZATION TO LACTOFERRIN

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Summary

This work was investigated the physicochemical study on lactoferrin and uptake process of immobilization silver to lactoferrin. A fast and simple methods of lactoferrin analysis are presented in this work. Spectrometric measurements of lactoferrin, its isoelectric point and electrophoretic analysis was applied for protein characterization. Nanocomlex of silver onto lactoferrin was obtained by binding of silver cations to lactoferrin. The infrared spectroscopic study and chromatographic study combined with spectrometric analysis were confirmed metal sorption process. Physicochemical description of immobilization silver to lactoferrin and was carried out such as a crucial point in its comprehension and potential applications in the field of medicine. The application studies were carried out using flow cytometry and antibiograms tests against selected clinical bacteria.

1 Introduction

Lactoferrin (lactotransferrin, LTF) is the protein of whey and the most useful building block for the synthesis of hemoglobin, plasma proteins and stimulate the proliferation lymphocytes, phagocytic activities of macrophages [1]. It is a glycoprotein, which contribute in transport and regulatory process of iron in cells. In milk, LTF occurs mainly in the form of globular micelle (biocolloids). Lactoferrin biocolloids have naturally tendency of metal binding, to form metalloproteins [2]. LTF is a precursors of many bioactive peptides. LTF-derived peptides are released during enzymatic digestion. They contribute in regulatory function of organism such as hormone like-activity, opioid agonist, mineral binding, antihypertensive activity and immuonomodulatory processes. Moreover, antimicrobial peptides of lactotransferrin can kill sensitive pathogenic microorganisms [3].

Silver cations are involved in many centers of active enzymes localized e.g. in nucleolus [4]. Therefore, they are used in many staining methods [4,5]. However, the major problem is toxicity of free silver cations. They may cause degradation of neurons, glia and all cells [5]. On the other hand, silver nanocomplex are widely used as antimicrobial, antiviral agents [6]. Therefore, there is a need to binding the silver cations with bioactive ligands, to eliminate its toxic properties for human cells and increased antimicrobial properties. Synthesis of silver nanocomplex based on lactoferrin creates a potential application in the field of medicine. Moreover, bioactive Ag-LTF-complex forms nutraceuticals and pharmaceutical applications.

The aim of this study, in the first step, was characteristics of LTF. In the next step, the goal was carried out the immobilization process of silver to lactoferrin and its physicochemical characteristics and confirmation. At the end, it was carried out the microbiological study using Ag-LTF nanocomplex.

2 Experimental

2.1 Materials

Lactoferrin was isolated from cow’s milk obtained from local dairy factory (SM Drzycim, Poland). The process of isolation and purification was carried out accoriding to Nuijens et al. methods (United States Patent No. 5861491).

2.2 Characteristics of lactoferrin

The isoelectric point of LTF was measured using Zestasizer Nano Series (Malvern Instruments, Malvern, Great Britain) using titration approach. The electrophoretic analysis was carried out using commercial system of gradient gel Bolt 4-12% (Novex, Life Technologies, USA) in Bis-Tris buffer system. The separation process was performed at 200V through 25 min.

Mass spectrometric analysis was conducted using an ultra-Xtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonik, German). Peptide mass fingerprint (PMF) spectra of tryptic digests of the casein components were recorded in refection positive mode, within an m/z range of 500–4000. MS spectra of intact proteins were obtained in the linear positive mode in an m/z range of 10 000–50 000.

2.3 Immobilization process

LTF (50mg/10mL) was suspended in 0.09% NaCl solution at pH 6. The solution was sonicated during 40 min. 1mL of lactoferrin suspension was transferred to 2mL Eppendorf tube. Next, 1mL of $AgNO_3$ basic solution with silver ions was added to the tube. After incubation, the solutions were centrifuged (4°C, 15000 rpm, 45min). 1mL of the supernatant was transferred to a Falcon probe and subjected to Millipore filtration with a molecular mass cut-off of 3kDa and then diluted with ddH_2O. Concentration of silver cations was determined by AAS (Atomic Absorption Spectroscopy) (Unicam, Great Britan).

2.4 Confirmation of binding process

Chromatographic separation was performed with the use of C18 home-made columns (125x4.6 mm) packed with materials based on 5mm silica particles. Mobile phase ACN: 0.1% TFA 50%:50%. Sample of LTF and Ag-LTF was suspended in ddH_2O. Fractions were collected manually every 30 s. Each fraction was evaporated on a Labconco CentriVap DNA concentrator (Kansas City, USA). Then obtained fraction was analyzed on MALDI TOF/TOF MS system using standard protocol in DAN mode.

The infrared spectrum of LTF and Ag-LTF was measured in MIR range (FTIR Genesis II Mattson, USA) using the thin layer methods on CaF_2 plates.

2.5 Microbiological study

Antimicrobial activity of silver nanocomplex onto lactoferrin was verified using flow cytometer methods according to manufacturer protocol (BD, Holland) and using classical antibiogram methods. Tested bacterial strain: *S.aureus*, *E.coli*, *B.subtillis*, MRSA and MSSA. All of them were from ATCC collection.

3 Results and Discussion

The isoelectric point (pI) of lactoferrin amounts 6 ± 0,5 (Fig.1A). The obtained pI values are somewhat inconsistent with reported data. This situation results from with different methods. pI values of 7.8 and 8.0 were reported for bovine lactoferrin by free-boundary electrophoretic method while the value of 4.8-5.3 was obtained by polyacrylamide gel IEF methods [7]. The obtained masses of LTF is 80kDa (Fig 1B).

Obtained data are consistent with proteins repositories and reported researches [8]. Concentration of silver in supernatant decreased with increasing incubation time. The kinetic curve was expressed by two different stages: the first one - initially rapid stage of silver adsorption, and the second stage with much slower silver immobilization approaching to equilibrium. The differences in retention time between LTF and Ag-LTF complex and differences in PMF spectra are proof of immobilization process. Moreover, the change in infrared spectra between LTF and Ag-LTF determine contribution of amino, carboxyl and imidazole group of aminoaced in binding process [9]. The results obtained from flow cytometry showed the antimicrobial properties against MRSA, MSSA and *S.aureus*. Ag-LTF complex

exhibited bacteriostatic and bactericide mechanisms with amoxicillin and metronidazole.

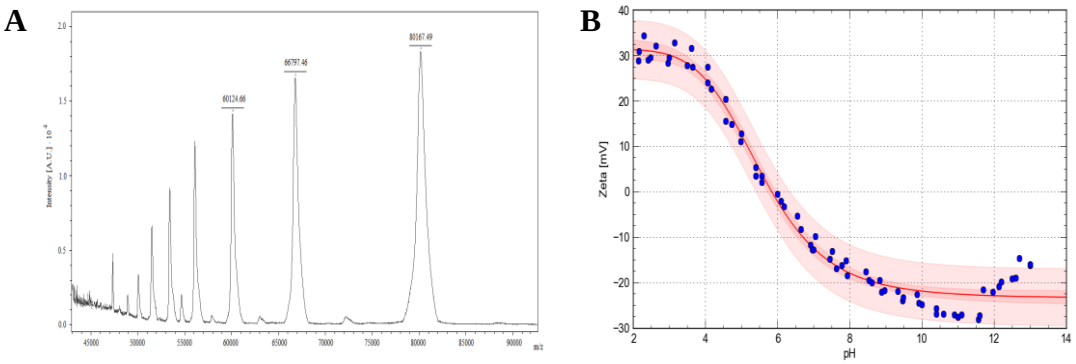

Fig. 1. (A) MALDI TOF MS intact spectra of LTF (B) Zeta potential of LTF particles in depends of pH values.

4 Conclusions

Present study manifested evidence that the silver ions should be effective uptake by native forms LTF from aqueous solution. The kinetic of the silver binding to protein is process heterogeneous process and is carried out in two stages: initial rapid stage with about 70% of the metal bound amount and significant slower second stage with approaching to equilibrium. Obtained results suggest perspectives, in which the nanocomplex of silver with lactotransferrin could be used in the field of medicine and food industry.

Acknowledgement

This work was supported by the National Science Centre (NCN, Poland) Grant No. 2013/08/W/NZ8/00701 (Symfonia-1) and No. UMO-2013/11/N/ST4/01835 (Preludium) as well as from grant "Step in the future" (KWP V).

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DEVELOPMENT AND APPLICATIONS OF IONIZATION TECHNIQUES IN AMBIENT MASS SPECTROMETRY

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1. Introduction

Ionization in ambient mass spectrometry carries out outside the machine in an open atmosphere. Nowadays, we recognize about 30 ionization techniques in the ambient mass spectrometry. They provide rapid analysis with no or only minimum sample preparation. Desorption electrospray ionization (DESI) [1] and desorption atmospheric pressure photoionization (DAPPI) [2] are the ionization techniques examined in this study. These methods employ solvent spray for desorption and ionization of analytes from the solid surfaces. The aim of this study was to develop a rapid analytical technique enabling to localize separated compounds on the TLC plate and secretory gland openings on the insect body surface and characterize compounds of the secretion using a setup combining DESI/DAPPI-MS with a software-controlled moveable motorized sample holder.

2 Experimental

2.1 TLC analysis

Six lipid standards and then vernix caseosa extract were separated on Nano Silica XHL HPTLC plates (Sorbent Technologies, Inc.). Developed TLC plates were mounted on software-controlled moveable motorized sample holder and spatial distribution of separated compounds was tracked. Lipid standards were detected by means of DESI, some lipid components of the vernix caseosa extract by means of DAPPI.

2.2 Insect analysis

Soldiers of the termite *Prorhinotermes simplex* and adult stink bugs *Graphosoma lineatum* were killed by freezing (–18 °C, 30 min), fixed on the glass slide using the correction fluid and mounted on software-controlled moveable motorized sample holder. Body surface was scanned by means of DAPPI to map the spatial distribution of defense compounds.

3 Results and Discussion

3.1 TLC analysis

Appropriate conditions for DESI analysis of different lipids representing different lipid classes after the separation on the TLC plate were found. DAPPI was utilized for

the detection of some lipid components of the vernix caseosa extract after the separation on the TLC plate.

3.2 Insect analysis

Examined model objects were two insect species, i.e. soldiers of the termite *Prorhinotermes simplex* and adult stink bugs *Graphosoma lineatum*. DAPPI in negative ion mode was used to map the spatial distribution of (*E*)-1-nitropentadec-1-ene, which is the biosynthesized defensive compound, on the body surface of *P. simplex* soldier. Opening of the frontal gland was localized. DAPPI in positive ion mode was used to track the spatial distribution of selected unsaturated aldehydes (previously described as the predominant components of the defensive secretion [3]) on the body surface of *G. lineatum*. Opening of the metathoracic scent glands was localized in the posterior part of the thorax.

4 Conclusions

Development of non-destructive imaging techniques suitable for the examination of TLC plates and biological samples was done.

Acknowledgement

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COMPARISON OF ANTIOXIDANT PROPERTIES OF DIFFERENT MENTHA PIPERITA SPECIES AND COMMERCIAL TEAS BY CAPILLARY ZONE ELECTROPHORESIS AND SPECTROSCOPY.

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Summary
A comparison of antioxidant properties of ten *Mentha* species and eleven commercially available peppermint teas was performed by electrophoretic fingerprinting of flavonoids and phenolic acids in aqueous infusions and by spectrophotometric determination of total polyphenolic content (TPC) and total antioxidant activity (TAA). Capillary zone electrophoresis with UV detection was used for measurement of fingerprints from the extracts. The antioxidant characteristics were determined by a spectrophotometric method using Folin-Ciocalteu, ABTS radical and DPPH methods. Significant differences were found among various *Mentha* species and also among different tea brands.

1 Introduction

Genus *Mentha* (peppermint) includes many species and hybrids that differ not only in plant anatomy but also in taste, flavour and chemical composition. Peppermint herbal preparations are mostly applied for treating rheumatism, dyspepsia, gastric discomfort and skin allergies. Most *Mentha* species have also antibacterial and antioxidant effects, which can be attributed to the presence of polyphenolic compounds and essential oils. *Metha x piperita* species is propably one of the most used ingredients in herbals teas [1]. It has been reported that extraction of essential oils from peppermint infusions is low and only about 21% of the original essential oils is extracted, while on the other hand up to 75% of the original polyphenolic content is extracted. For this reason, our work focused on fingerprinting of polyphenolic compounds that are more stable during boiling and storage [2] and thus more bioavailable.

The interest in clarifying the presence and function of biologically active compounds in plants is growing and requires the development of new analytical methods. Capillary zone electrophoresis (CZE) belongs to the most applied separation techniques for the determination of polyphenols in herbal material. Prior to the CZE analysis however, the most important part of the method development is sample preparation. In our work, various extraction procedures were used for sample preparation and the most suitable treatment was applied to ten samples of *Mentha* species and eleven samples of peppermint teas. Further, all samples were evaluated

for the total antioxidant activity (TAA) and total phenolic content (TPC). The main goal of this work was to compare the information obtained from the electropherogram fingerprints with the spectrophotometric analysis (TPC, TAA) in various Mentha species. Further goal was to evaluate the antioxidant properties of commercially available peppermint teas.

2 Experimental

The measurements were performed using a HP 3D capillary electrophoresis system (Agilent, USA). Separation was carried out in a 50 μm ID fused silica capillary of total length 68.5 cm. The separation buffer was 20 mM borate buffer with 10% methanol. The separation voltage was 20 kV and the samples were injected hydrodynamically at a pressure of 50 mbar for 10 seconds. The detection was done at 254 nm [3].

The antioxidant characteristics were measured spectrophotometrically using a Unicam 5625 spectrophotometer equipped with a quartz cuvette, 10 mm optical path and all results were expressed as gallic acid equivalent (GAE).

3 Results and Discussion

The mixtures of dried leaves, stalks and blooms collected from various *Mentha* species were milled to powder. Different extraction methods were developed for sample preparation. In the first extraction method, boiling water was poured to the milled sample and extracted for 6 min, followed by centrifugation at 2500 x g for 5 min and filtration through a 0.22μm Nylon filter. In the second extraction method, samples were mixed with 50% methanol and put to the ultrasonic bath for 15 min at room temperature, centrifuged and filtered as mentioned above [4]. Part of the water infusion was extracted with ethylacetate (l-l extraction). Similarly, part of the methanolic extract was extracted with diethylether. The prepared extracts were analyzed using CZE and served for the determination of antioxidant properties. The remaining aliquots of samples were stored in a freezer at -18 °C [5].

Electrophoretic analyses focused on comparison of total amount of extracted compounds (total sum of peaks) with selected analytes from the group of flavonoids. Figure 1 shows the electropherograms of constituents of three different *Mentha* species extracted with method 1 (boiling water). For comparison, Figure 2 shows electropherograms of peppermint tea aqueous extracts using the same method.

The total polyphenolic content (TPC) was determined using Folin- Ciocalteu method. Total antioxidant activity (TAA) was measured using scavenging of ABTS cation radical and DPPH method. The obtained values show noticeable differences between individual samples but also between applied extraction procedures. The resulting values of TPC and TAA are expressed as gallic acid equivalent (in mg GA/g of dry samples).

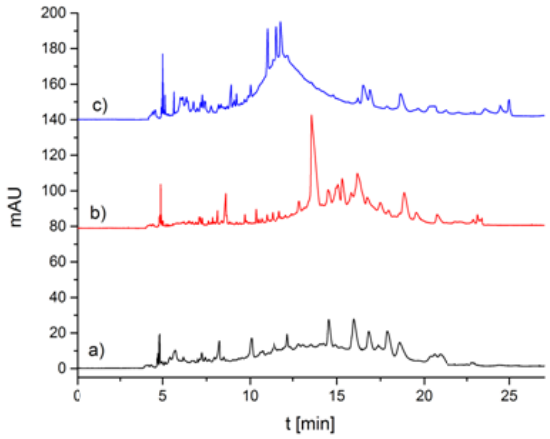

Fig. 1. Electropherogram of the water extracts: a) *Mentha x piperita*, b) *Mentha x piperita citrata*, c) *Mentha x piperita grapemint.*

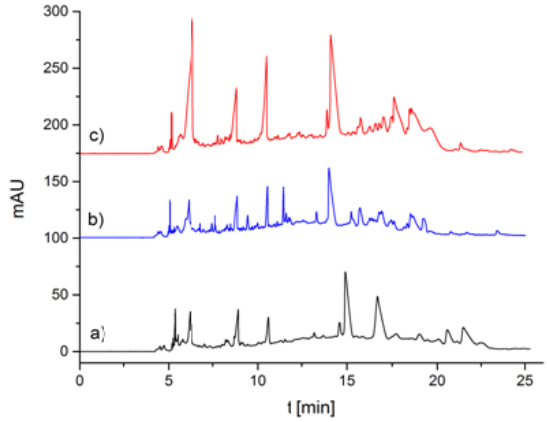

Fig. 2. Electropherogram of the water extracts of peppermint teas: a) Babička Růženka, b) Pickwick, c) Bio Jardin.

The diffences of TPC and TAA between individual *Mentha* species are shown in Table 1. The hightest content of polyphenols and the hightest antioxidant activity was determined in *Mentha x piperita swiss ricola*. Except *Mentha longifolia*, the values of TPC for the rest of samples were about half of TPC value of *M.P. swiss ricola*. On the other hand and in spite of the wide range of TPC values, the differences between TAA values are not so eminent. The only exception was *M.P. multimenta,* which showed the lowest content of polyphenolic compounds and correspondingly also the lowest TAA values. This was confirmed by both spectrophotometric measurements and CZE analysis. In the tested peppermint herbal teas, a wide range of values for both antioxidant characteristics (TPC and TAA) was determined. Interesting observation was that the highest values of TPC or TAA were obtained for Lord Nelson which is the one of the cheapest peppermint teas.

Table 1. The antioxidant characterictics of *Mentha* species and peppermint teas water infusions.

Mentha species	TPC[a]	TAA[a]		Peppermint Teas	TPC[a]	TAA[a]	
	Fol.-Cio.	DPPH	ABTS		Fol.-Cio.	DPPH	ABTS
M. longifolia	52,52	16,64	8,31	Lord Nelson	68,29	19,84	44,56
M. aquatica	43,52	16,42	14,73	Estonia Mint	66,65	19,60	54,43
M.S. applemint	43,92	16,73	10,83	Herba	61,93	19,84	41,76
M. piperita	27,32	13,72	5,36	Pickwick	55,20	20,19	29,49
M.P. chocolatemint	30,72	15,55	12,09	Bio Jardin	51,02	19,46	21,23
M.P. citrata	34,12	14,85	12,20	Mistrin Mint	47,20	17,91	21,09
M.P. grapemint	34,52	17,02	12,41	Sweet Mint	31,02	18,29	20,29
M.P. proserpina	31,92	14,54	5,99	Teekanne	24,29	13,84	10,83
M.P. multimenta	21,32	7,96	5,15	Megafyt	21,56	13,06	13,89
M.P. swiss ricola	75,92	17,14	12,94	Tesco Infusion	17,20	8,64	12,03
				Babička Růženka	15,38	5,86	4,56

[a] [mg GAE/g]

4 Conclusions

In this work various extraction methods were developed and compared with respect to the antioxidant properties of the final extract. Although methanolic extracts showed higher extraction efficiency, using boiling water extraction was preferred because it is of interest for the final consumer. Comparison of electrophoretic fingerprints showed significant difference between different *Mentha* species and also between different commercial tea samples. A good correlation between the total peak area from CZE analysis and TPC/TAA obtained spectrophotometrically was achieved. The knowledge about plant phytochemistry and antioxidant properties helps to understand its medical effects and helps streamline its utilization in natural medicine.

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TOWARDS CYTOCHROME P450 IMER FOR KINETICS AND INHIBITION STUDIES USING CAPILLARY ELECTROPHORESIS IN ONLINE CONFIGURATION

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Summary
The enzyme superfamily of cytochromes P450 (CYP) plays a key role in xenobiotic biotransformation and thusly in drug metabolism. Therefore assessment of the interaction between candidate drugs and CYP isoforms is an integral part of preclinical stage of the drug development process, where high throughput and cost effectiveness is a priority. Capillary electrophoresis (CE) offers a number of features which are suitable for these purposes, those being the low sample consumption, short analysis time and parallel analyses using multicapillary systems, what more integration of immobilized enzyme microreactor (IMER) into the separation capillary enables full automation and repeated use of the enzyme. A new online method for inhibition studies based on cytochrome P450 2C9 isoform IMER integrated into CE system is thus presented.

1 Introduction

Large numbers of potential drugs are tested every year in preclinical stages of their development and as was mentioned CYP inhibition screening represent a major part of those studies. Nevertheless current methods based on fluorescent probes are expensive and inefficient, enormous effort is therefore put into development of new high throughput cost effective methods. Immobilization of CYP enzymes represents promising tool offering enzyme recycling and therefore cost reduction. Regrettably the CYP enzymes are membrane bound which makes their immobilization rather challenging and displayed properties like stability and activity are mostly inferior in comparison to immobilized soluble enzymes [1]. In this work we present general procedures and techniques that were used to overcome above mentioned drawbacks towards developing a novel method for kinetics and inhibition studies.

2 Experimental

2.1 Immobilization procedure

Microsomes containing recombinant CYP2C9 (CYP2C9 BACULOSOMES® Plus Reagent, rHuman, Thermo Fisher Scientific Inc.) were immobilized on magnetic microparticles Si-MAG-Carboxyl (diameter 1 µm, Chemicell GmbH). Solutions of all reactants were prepared in immobilization buffer (30 mM potassium phosphate, pH 8.60). Microparticles were washed in immobilization buffer and then activated by 625 µM N-hydroxysulfosuccinimide and 375 µM 1-ethyl-3-(3-dimethylamino-

propyl)carbo-diimide while slowly mixing for 2 hours at 25°C. After the activation, the microparticles were washed again and the solution of CYP2C9 microsomes containing 0.32 $mg \cdot ml^{-1}$ of total protein, 40 µM flurbiprofen and 40 µM dapsone was added and immobilization was achieved by slowly mixing for 20 hours at 5°C. The gained microparticles were washed carefully after the immobilization process in incubation buffer (50 mM TRIS, pH = 7.50 adjusted with HCl) and then diluted in storage buffer prepared from incubation buffer containing 20 % glycerol and stored at -70°C.

2.2 In-capillary reaction

The solution of prepared microparticles with attached microsomal CYP2C9 was thawed, washed with water and injected into capillary, where they formed an IMER in the magnetic field created by two NdFeB magnets. The reaction was started by introduction of a plug of incubation mixture containing a certain concentration of diclofenac and 1 mM NADPH in incubation buffer. The incubation was carried out at 30°C for 15 minutes and terminated by application of voltage causing the reactants leave the reactor.

2.3 Separation

All the experiments were performed on the Agilent 7100 CE System. An uncoated silica capillary (total length 33.5 cm, effective length 19 cm, 75 µm i.d.) was thermostated at 30°C and the background electrolyte (BGE) consisted of 100 mM TRIS and 80 mM boric acid. Separations were accomplished by application of 15 kV, positive polarity.

3 Results and Discussion

Parameters of immobilization were adapted from previous research in our group. Cassette of CE system was modified to hold two NdFeB magnets in two possible arrangements. During the method development, number of parameters was optimized in order to measure enzyme kinetics and inhibition, main benefits were brought with magnets rearrangement, incubation buffer optimization and data processing. The magnets rearrangement brought significant improvement considering IMER constitution resulting in overall activity repeatability increase; see figure 1.

Incubation buffer and BGE were changed from phosphate based to TRIS based buffers offering good separation at lower voltage while conserving sufficient enzyme activity resulting in significant increase of operational stability of the enzyme; see figure 2.

Persisting activity loss was compensated using data processing and sequence arrangement. Acquired data were used to calculate Michaelis constatnt for diclofenac conversion by CYP2C9 and later for characterization of model inhibition of this reaction by sulfaphenazole. All results were in very good agreement with data found in literature obtained with free microsomal CYP2C9 proving that prepared method is suitable for enzyme kinetics and inhibition studies. What more the amount of reagents was significantly smaller compared to the most advanced conventional methods [2],

for example the amount of CYP2C9 per analysis is lower by up to two orders of magnitude, depending on calculation method.

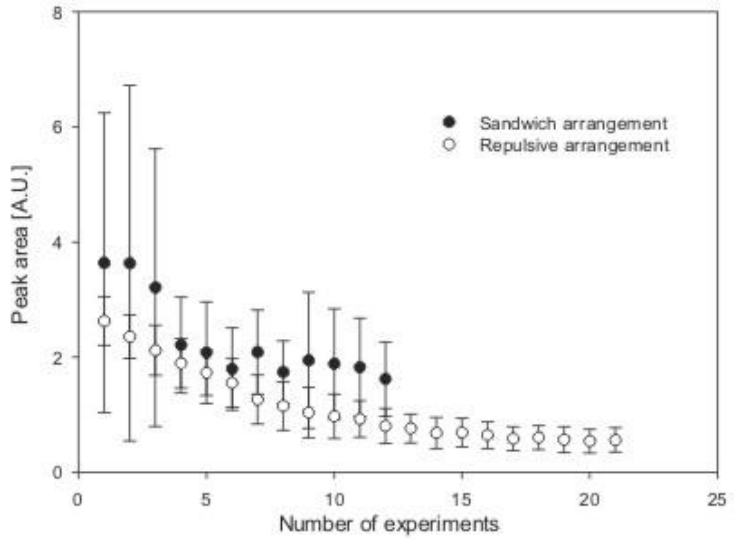

Fig. 1. Effects of magnet arrangement on IMER operational stability a repeatability of its activity.

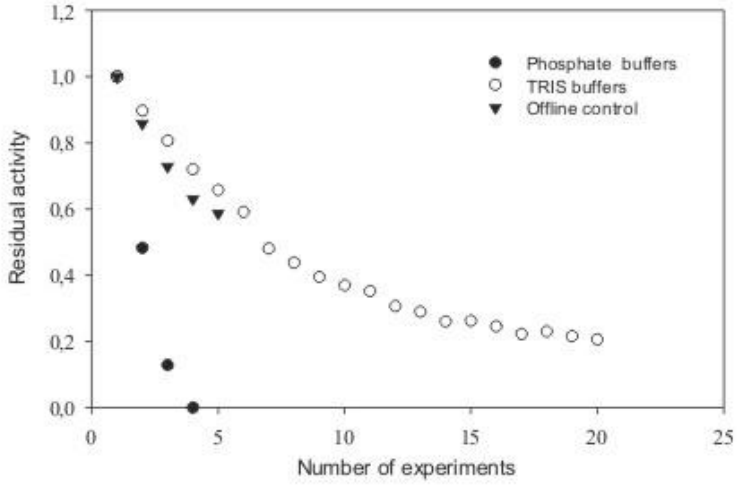

Fig. 2. Effect of buffer composition on IMER operational stability.

4 Conclusions

With careful optimization and simple data processing we were able to obtained valid results and significant savings of reagents using immobilized microsomal CYP2C9. Furthermore a simple transfer to multicapillary systems would immensely increase the method throughput making it suitable for rapid online screening of CYP2C9 inhibitors.

Acknowledgement
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WATER UPTAKE ON SILICA-BASED STATIONARY PHASES IN HYDROPHILIC INTERACTION CHROMATOGRAPHY

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Summary
Excess adsorption of water from organic rich mobile phases was investigated on sixteen stationary phases using the frontal analysis method and coulometric Karl-Fischer titration. Preferential adsorption of water on columns tested can be described by Langmuir isotherms on all columns studied. Less than one monomolecular water layer equivalent was adsorbed on moderate polar silica hydride-based stationary phases, Ascentis Express F5 and Ascentis Express CN column at the saturation capacity, while on more polar stationary phases, several water layer equivalents were up-taken from the mobile phase. The strongest affinity to water was observed on the ZIC cHILIC stationary phases, where more than nine water layer equivalents were adsorbed onto its surface at its saturation capacity. Significant correlations were found between the water uptake and the separation selectivity for compounds with strong polarity differences.

1 Introduction
The aqueous normal phase liquid chromatography is believed to be less affected by the solvent demixing effect than the non-aqueous normal phase LC. As postulated by Alpert in 1990, water is preferentially adsorbed from mixed aqueous-organic mobile phases, and forms a diffuse water-rich layer at the surface of the adsorbent [1]. This HPLC mode is also known as Hydrophilic Interaction Liquid Chromatography (HILIC), in which the retention is supposed to be largely controlled by partition between the bulk mobile phase and the adsorbed water-rich layer. However, because of the miscibility of polar organic solvents and water, no fixed boundary between the

two liquid phases can be fixed in the column. The retention may be also contributed by the adsorption of the solute onto the surface of a polar stationary phase In the present work, we investigated the excess adsorption of water in the ANP mode on various types of polar and non-polar stationary phases by frontal analysis, to compare the formation of water-rich layer on the surface of the stationary phases with different functionalities and the role it may play in the ANP retention mechanism. The present study extends our recent work on hydrosilated silica based columns.

2 Experimental

Before each measurement, the column was rinsed with 50 column hold-up volumes of acetonitrile. The data necessary for the determination of excess concentration of water, q_{ex}, adsorbed onto the surface of the columns tested were measured using the frontal analysis method for twelve feed solutions of water (0.5% – 15.0%), in acetonitrile. The column temperature was kept constant at 40°C. For each feed solution, the concentration of water in the collected effluent fractions was measured by coulometric Karl Fischer titration, and the data were used to construct the breakthrough curves, the dependence of water concentration in each fraction on the volume of mobile phase passed through the column. From the inflection points of the breakthrough curves, the breakthrough volumes, V_B, were evaluated and the excess concentration of water adsorbed on the stationary phase, q_{ex}, was calculated for each concentration of water in the mobile phase, c_m, as $q_{ex} = \{(V_B\text{-}V_M)\cdot c_m\}/V_i$, where V_M is the column hold-up volume and V_i is the volume of inner pores). The approach was repeated in triplicate for each feed solution of water in acetonitrile. The data were used for the construction of excess water distribution isotherms showing the dependence of concentration of excess water adsorbed on the stationary phase, q_{ex}, on the concentration of water in the mobile phase, c_m (Fig. 1). Both concentration of excess water in the stationary phase and concentration of water in the mobile phase are expressed as volume fraction of water.

3 Results and Discussion

On each column, we determined the excess water uptake from acetonitrile-rich aqueous-organic mobile phases by frontal analysis method. All plots on the columns studied have similar profiles with typical sigmoidal shape, in which the inflection point corresponds to the breakthrough volume, V_B, from which the excess concentration of water retained in the stationary phase was determined. As usual, the breakthrough volumes, V_B, decrease with increasing concentration of water in acetonitrile/ water mobile phases.

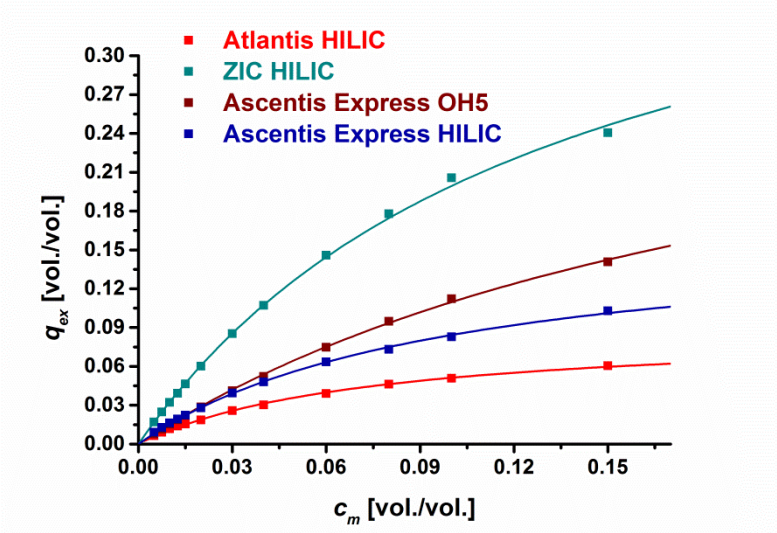

Fig. 1. Experimental isotherm profiles of some stationary phases tested.

The amount of water in the stationary phase was expressed as the volume fraction of excess water contained in the inner pore volume. Using this convention, q_{ex} is the water concentration contained in the pores in excess to the bulk mobile phase concentration, c_m. Fig. 1 shows some experimental isotherm profiles which correspond to classical Langmuir adsorption model, described by Eq. (1), where K_{D0} and b are characteristic constants for each stationary phase tested [2].

$$q_{ex} = \frac{K_{D0} \cdot c_m}{1 + b \cdot c_m} \qquad (1)$$

The constant K_{D0} corresponds to the distribution constant of water in the pores of the stationary phase at very low c_m, and the constant b is related to the concentration of water under saturation conditions, $q_{satur} = K_{D0}/b$ (the column saturation capacity). The isotherm profiles show relatively good validity of the Langmuir model for the water uptake on the silica columns. From the column saturation capacity data, the equivalent number of adsorbed monomolecular water layers inside the pore volume at full saturation capacity, can be estimated as:

$$N_w = \frac{q_{satur} \cdot V_i \cdot N_{AV} \cdot A_{H2O} \cdot \rho_{H2O}}{A_{s(silica)} \cdot \rho_{silica} \cdot V_{column} (1 - \varepsilon_T) \cdot f_{core} \cdot Mr(H_2O)} \qquad (2)$$

where V_i is the pore volume mL, N_{AV} is the Avogadro constant, $6.023 \cdot 10^{23}$, A_{H2O} = $18.9 \cdot 10^{-16}$ cm^2 is the area occupied by a molecule of water adsorbed on the flat adsorbent surface, calculated from the van der Waals atom radii; ρ_{H2O} is the density of water, 1 g/cm^3; A_s is the specific surface area of silica adsorbents according to the manufacturer's data), ρ_{silica} = 2.3 g/cm^3 is the density of silica, V_C is the column volume, ε_T is the total column porosity, f_{core} is core shell factor and $M_r(H_2O)$ = 18.015 g/mol is the molar mass of water. N_w does not represent the real number of water layers adsorbed on a surface of stationary phase, but it just enables a comparison of up-taken excess water. The phosphorylcholine zwitterionic stationary phase, ZIC

cHILIC, shows the N_w equivalent to more than nine monomolecular water layers due to its enhanced affinity to water; followed by second zwitterionic sulfobetaine ZIC HILIC phase with the equivalent of six adsorbed water layers. Equivalent from three to five monomolecular water layers were observed on the stationary phases with bonded hydroxyl groups, Luna HILIC (4.72), YMC Triart diol (3.06) and Ascentis Express OH5 (3.98). Between one and two monomolecular water layers equivalents are adsorbed on XBridge HILIC, Atlantis HILIC, Ascentis Express HILIC and LiChrospher diol columns. The less-polar Ascentis Express ES-CN column shows very low water saturation capacity, like the hydrosilated Cogent columns.

4 Conclusions

The adsorption isotherms of water can be described by Langmuir model, like in non-aqueous normal-phase LC. At full column saturation, the excess adsorbed water fills 2.3 – 45.3% pore volume, which approximately corresponds to the equivalent of 0.25 – 9 water layer coverage of the adsorbent surface. It is assumed that the equivalent number of adsorbed water layers decreases with decreasing number of active silanols and hydroxyl groups. In the ANP mode, partition retention mechanism is probably the predominating retention mechanism on the stationary phases with higher number of water-layers, while the columns with less than one equivalent of water layer support the idea of predominating role of adsorption retention mechanism.

Acknowledgement

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GRAPE POMACE APPLICATION IN ENVIRONMENTAL STUDIES: FROM WASTE TO NATURAL FOOD PRESERVATIVE AND SOURCE OF BIOFUEL

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1 Introduction

The geographic location of Republic of Macedonia is exceptional for breeding vine and specific grape varieties. But, the wine industry waste in general is a problem in

Macedonia, since it does not have any usage. In the European Union, there is approximately 14.5 million tons of wine industry waste produced from wineries (http://www.academicwino.com/2012/11/grape-seed-extract-leather-production.html). In fact, the wine industry waste (grape pomace) contains primarily crushed grape skins and seeds rich in beneficial polyphenol compounds that act as antioxidants, antibacterial agents etc. The largest fraction of winery waste is pomace, or the solid remains of grapes (skins, stalks and seeds), which is thrown away ending up in landfills. From another point of view, transport is the third largest emitter of greenhouse gases and biofuels can significantly reduce transport's carbon footprint since it is dependent on finite fossil fuels such as oil and petroleum for its energy needs (R.E.H. Sims, et al.). Biodiesel, provides significantly reduced emissions of carbon monoxide; compared to petroleum diesel fuel.

2 Experimental

Totally, 4 types of Macedonian grape pomace, (from Zupjanka, Prokupec, Kadinal and Vranec varieties) as well as blueberry and aronia, were used. For the extraction of polyphenolics liquid-liquid extraction with ethanol/water/acetic acid, followed by decantation and filtration was used. The phenolic content of the obtained extracts was determined by the Folin-Ciocalteu method (Ivanova et al. 2010); and the total anthocyanins were realized by the Di Stefano et al. (1989) method. Afterwards, three different volumes of the obtained extracts (10, 50 and 100 mL) from each sample, were concentrated by rotoevaporation to dryness. The three concentrates were applied on milk together with the lactic bacteria to study the influence of polyphenolics during fermentation; as well as, applied on milk after the fermentation (into the obtained yoghurt) (Figure 1). The pH value of the newly generated yoghurts was observed by using a pH meter. In the second part, the seeds were separated from the grape pomace and served as a new source of oil, together with blueberry and aronia peels, that was to be transformed into biodiesel. Six organic solutions (ethanol, hexane, benzene, diethyl ether, acetone, acetic acid) were added to the dried seeds and then filtration and distillation followed for obtaining the oil. By transesterification reaction the biodiesel was divided and it was proved due to the combustion of the final product.

3 Results and Discussion

Fig. 1. Yoghurt enriched with polyphenols, from Aronia, added after fermentation.

Table 1. Comparison of the total phenols in the analysed samples (grape pomaces and berries).

Total phenolic assay (TP)		
Name of sample analyzed	**TP (mg/L)**	**TP average value**
1 – Zupjanka	62.8	67.9
	73.17	
2 – Kardinal	76.17	76.7
	77.21	
3 – Prokupec	247.01	247
	246.03	
4 – Vranec	152.18	152
	151.56	
5 – Blueberry	190.42	192
	194.21	
6 - Aronia	430.2	431
	432.49	

Table 2. The pH variation between some of the samples.

pH values of yoghurt with polyphenols added during fermentation								
Polyphenols added	**31.01**	**02.02**	**04.02**	**06.02**	**09.02**	**15.02**	**22.02**	**02.03**
Zupjanka								
1-10	4.0	4.0	4.0	4.0	3.9	3.9	3.9	3.9
1-50	4.3	4.0	4.0	4.0	3.9	3.9	3.9	3.9
1-100	4.2	4.0	4.0	4.0	3.9	3.9	3.9	3.9
Kardinal								
2-10	4.1	3.8	4.0	3.8	3.8	3.9	3.9	3.9
2-50	4.2	3.9	4.1	3.9	3.9	3.9	3.9	3.9
2-100	4.0	3.9	4.0	3.9	3.9	3.9	3.9	3.9
Prokupec								
3-10	3.9	3.7	3.9	3.9	3.9	3.9	3.9	3.9
3-50	4.1	3.8	3.9	3.9	3.9	3.9	3.9	3.9
3-100	4.2	4.0	4.1	4.0	4.0	4.0	4.0	3.9
Vranec								
4-10	4.0	3.8	3.8	3.8	3.8	3.8	3.9	3.9
4-50	4.1	3.9	3.9	3.9	3.9	3.9	3.9	3.9
4-100	4.2	4.0	3.9	4.0	4.0	4.0	4.0	4.0
Blueberry								
5-10	4.0	3.7	3.9	3.9	3.9	3.8	3.9	3.9
5-50	4.1	3.9	4.1	4.0	4.0	3.9	3.9	3.9
5-100	4.2	4.0	4.01	4.0	4.0	4.0	4.0	3.9
Aronia								
6-10	4.0	3.7	3.9	3.9	3.9	3.9	3.9	3.9
6-50	4.1	3.8	4.0	3.9	4.0	3.9	3.9	3.9
6-100	4.1	3.8	4.0	4.0	4.0	4.0	4.0	3.9

Table 3. Comparion in the oil and biodiesel (biodiesel + byproducts) obtained from the samples.

Name of sample (source of oil)	The amount of oil obtained (g)	Impure biodiesel obtained (g)
1 + ethanol	0.26	0.39
2 + ethanol	0.28	0.4
3 + ethanol	0.19	1.19
4 + ethanol	0.15	0.26
5 + ethanol	0.29	0.28
6 + ethanol	0.29	1.96
1 + acetone	0.2	0.32
2 + acetone	0.26	0.24
3 + acetone	0.36	0.43
4 + acetone	0.25	0.58
5 + acetone	0.13	2.43
6 + acetone	0.19	4.23
1 + acetic acid	0.28	1.54
2 + acetic acid	0.6	1.7
3 + acetic acid	0.31	3.72
4 + acetic acid	0.11	1.14
5 + acetic acid	0.29	4.54
6 + acetic acid	0.27	1.83

The dry phenolic extracts were applied on a home - made, traditional yoghurt during and after fermentation, to observe the change in the pH value (if there is any) of both types and of the three different concentrations. As it is already known, the varieties in the pH value of the yoghurt are directly proportional to the time of spoilage. Actually, a faster decrease in the pH values, indicates faster spoilage and lower shelf-life. With the help of polyphenols from the winery waste there should be a lower pH drop and a deceleration in the mold development in the new yoghurt. *There is already a dairy in Macedonia, "Buchen Kozjak" that is interested in taking the method from this project as a base for manufacturing of a new yoghurt, in our country.* Regarding the second part of the project, it is known that a larger amount of oil is present in the grape seeds, than in the stem and the peels. Five different organic solvents for extracting the oil contained in the seeds were applied. Later, it was observed that diethyl ether, hexane, and bensene had evaporated without extracting the oil. By use of transesterification method the oil obtained in 18 different amounts was converted into biodiesel and also some byproducts, soap, glycerol, excess alcohol, and trace amounts of water. Then the content was heated again until it stopped boiling, meaning that the byproducts were removed and biodiesel + glycerol was the only liquid left. The presence of biodiesel was proved by the 90% combustion of the remaining substance in all beakers.

4 Conclusions

All yoghurt samples containing polyphenolics applied before the fermentation, presented higher pH value compared to the control and samples with polyphenolics applied after fermentation. Zupjanka has shown the best results (pH-4.4 for 1-100) for all concentrations, which is even higher than aronia, which is known to contain the highest phenolic content. After obtaining the yoghurt, sensory analysis was performed, stating that the new product has creamy texture; it tastes good, without unpleasant smell or bitterness. The colour of some samples turned into red-violet, excluding the yoghurt with dry extracts from: Zupjanka and Kardinal (white grapes). By microbiological analysis the presence of pathogen and other harmful bacteria was proved to be negative, showing that this yoghurt could be a main basis for manufacturing a more beneficial dairy product that could be soon available on the market. Concerning the biodiesel production, the oil content, depending on the solvent used, differed from 0.11 g to 0.36 g. During the extraction acetone and acetic acid showed better results than ethanol, which proved to be a better extractor of antioxidands. Thus, acetic acid was the most proper solvent for obtaining impure biodiesel with a yield of 90.8%. Overall, the sample which showed the highest percentage when mixed with acetic acid was blueberry. On the other hand, from the grape pomace, the type comprising the largest amount of impure biodiesel was aronia with the average yield of 53.46% and then followed: blueberry, Prokupec, Kardinal, Zupjanka and Vranec, respectively.

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Abstracts of poster presentations

P1 ANALYSIS OF SEQUENCE SPECIFIC INTERACTIONS BETWEEN DNA AND P53 FAMILY PROTEINS BY ELISA, SLOT-BLOT AND EMSA

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Summary
DNA-protein interactions of core domains of p53, p63 and p73, members of tumor suppressor p53 family, were investigated by multiple methods with regard to verifying the sequence specificity with which short target oligonucleotides/long DNA fragments can be recognized. The sequence specificity of core domains and wtp53 full protein binding to specific sequence in both types of DNA substrates was confirmed in solution, on surface and in gels by ELISA, slot-blot and EMSA.

1 Introduction

Common division of DNA-protein interactions falls into two categories: sequence specific and sequence non-specific. Both protein-DNA binding sub-types have common fundamental means in electrostatic interactions, hydrogen bonding, hydrophobic interactions and base stacking. These forces contribute to proteins binding in varying combinations due to the actual substrates in use and affect the local differences in sequence specific and non-specific interactions. The relative specificity of DNA-protein interactions is thought to be involved in many biological processes.

Traditional approaches for DNA-protein detection include EMSA, filter assay, ELISA, reporter assays, ITC, SPR, FISH, ChIP, X-ray crystallography and more. Each approach has its own strengths and weaknesses concerning sensitivity, specificity and the very nature of the thing tested [1]. Recently, EMSA and imunoprecipitation techniques were was used for detection of mutant p53 and p53 family interaction with long DNA [2, 3]. P53 core domain sequence-specific binding to DNA was detected also by electrochemistry, complex p53 with DNA results in a striking decrease in the electro-catalytic signal of free p53 [4]. In our experiments the sequence specificity of interaction of p53, p63 and p73 was studied by EMSA, Slot-blot filter assay and ELISA which were subjected to optimization.

2 Experimental

2.1 ELISA

In-house streptavidin coated 96-wells imuno plates were prepared by incubating 100 µl 5 µg/ml streptavidin in 1xPBS for a week, 2x washed, and 2 hour incubation in 3% BSA, 2x washed. In the experiments a total volume 50 µl/well of on ice pre-incubated mixes 1x TETKD 0.5% BSA for 30 min RT with 5 pmol biotinylated DNA and varying protein + primary antibody (p53 family member + 400 ng DO-1 or anti-GST) solution is then added and incubated for 30 min in 4 °C, and later 4x washed. Afterwards 50 µl anti-mouse (1:1000) horseradish-peroxidase conjugated antibody was added and incubated for 1 h and later 4x washed. 50 µl TMB substrate was added and absorbance measured at microplate reader at 370 and 655 nm (Biotech). All washing steps were done with ice-cold 1x PBST + 0.5 %BSA.

2.2 Slot-Blot

48-well Slot-Blot apparatus was used with nitrocellulose (top) (Pall) and Hybond-N+ (bottom) (Amersham Bioscences) membranes. Membranes were pre-treated for 10 min in KOH, then 3x washed in water, and again pre-treated in 1x TETKD for 1h. Samples applied in total volume of 100 µl were 2x washed by 500µl ice-cold PBS. FITC-labelled oligonucleotides were then detected on Typhoon scanner.

2.3 EMSA, supershift, blotting

Reaction mixtures with protein and DNA were incubated for 20 min on ice before loading on gel. Acrylamide gels for protein-oligonucleotide interactions were 6% AA, 0.5x TBE, 10 mM KCl and run at 100 V for 50 min. Agarose gels for protein interactions with DNA fragments were 0.33x TBE, 1 % agarose, run at 90 V for 60 min. Gels blotted on nitrocellulose membrane (Pall) at 150 mA for 90 min.

3 Results and Discussion

3.1 Sequence specific binding of p53 family to DNA by EMSA and supershift assay

The most common method for DNA-protein interaction analysis is EMSA. Both acrylamide and agarose gels were used to study sequence specific interaction of p53 family proteins [2-5] with their recognition sequence P1 (in oligonucleotides or 495 bp long fragment DNA) (Fig.1). To validate specificity of interaction supershift assay was performed; DNA-protein complex was incubated with antibody as shown on Fig.1 for GSTp63CD complex with long DNA. We observed that anti-GST antibody did not disrupt the interaction of protein with DNA and than it can be used for other techniques such as ELISA. Both EMSA and antibody supershift assays confirmed that studied proteins bound specifically to DNA.

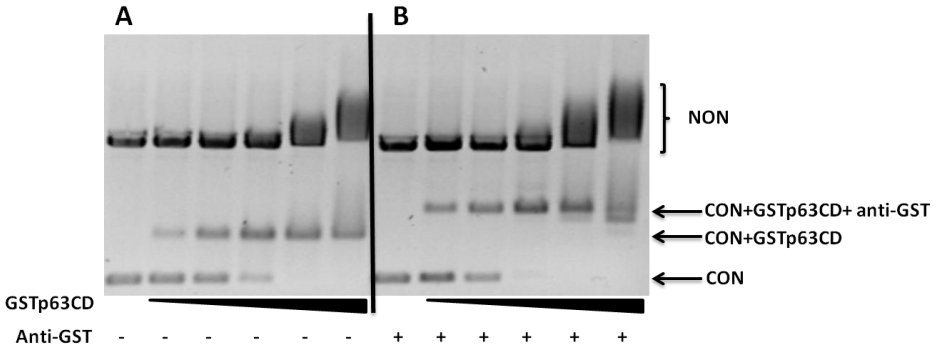

Fig. 1. EMSA analysis of anti-GST antibody supershifted p63-DNA complex. DNA (200ng pPGMI/PvuII fragments, shorter one contains p53 recognition site) was incubated with increments of bacterially-produced, purified p63 protein (GST-p63CD). The specificity of EMSA signal was verified by adding 200 ng anti-GST antibody (SIGMA)- part B. DNA and DNA-protein complexes were detected with EtBr.

3.2 Sequence specific and non-specific binding of p53 family by ELISA

To study p53 family interaction with DNA immobilized on surface ELISA assay was optimized [6]. Proteins of interest with specific primary antibody were bound to biotinylated oligonucleotides of the same length containing specific recognition site for p53 family (P1) and/or $(dA)_{50}$ immobilized on a microtiter plate. Complexes of DNA-proteins were detected by colorimetric assay with horseradish labeled secondary antibodies after washing away unbound protein fractions. Washing steps and amounts of proteins, DNA and antibody were optimized to obtain sequence specific interaction of p53 family proteins to P1 oligonucleotide.

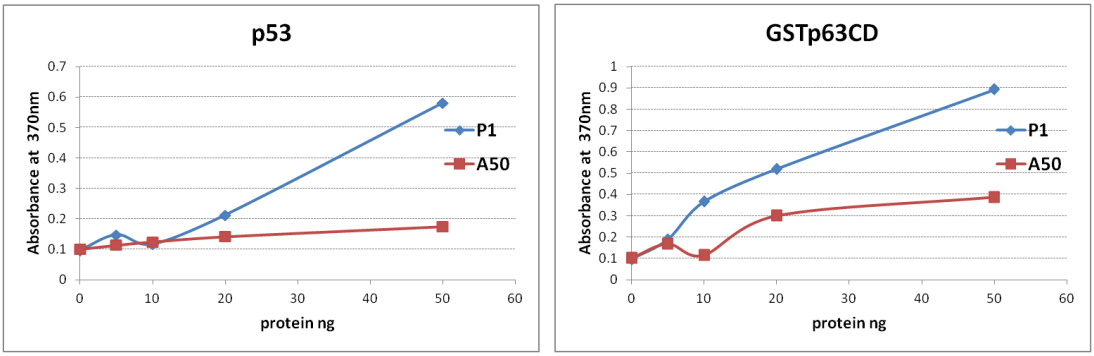

Fig. 2. p53 and GSTp63CD prefer sequence specific binding to P1 substrate to non-specific binding to A_{50} oligonucleotide.

3.3 Sequence specific and non-specific binding of p53 family by slot-blot

To study interaction of p53 family proteins with DNA in solution slot-blot assay was performed. Fluorescently labeled oligonucleotides P1 and $(dA)_{50}$ were mixed with proteins and applied to slot-blot apparatuses. Slot-blot consists of two membranes; top for recovery of DNA-protein complexes and bottom to capture the free DNA probe. After DNA protein mixture has been applied on upper membrane, unbound DNA was

thoroughly washed by buffer and the interaction strength/likelihood of p53 family proteins to P1/$(dA)_{50}$ was detected.

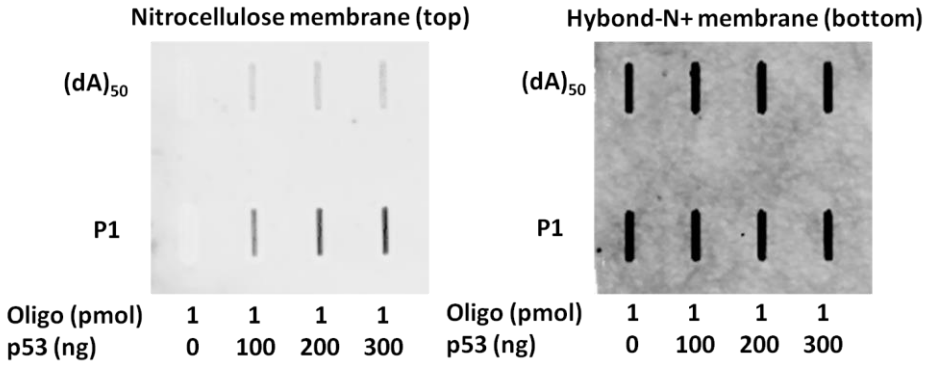

Fig. 3. Oligonucleotide containing specific sequence (P1) was trapped in protein-DNA complex to greater extent than non-specific oligonucleotide d(A50). FITC-labeled oligonucleotides were either trapped with protein on nitrocellulose membrane or were washed through and captured on the latter Hybond-N+ membrane.

4 Conclusions

P53 family proteins interaction with sequence specific and non specific substrate was studied in gel, immobilized on surface and in solution by EMSA, ELISA and slot-blot assays. Each approach has its own strengths and weaknesses concerning sensitivity, specificity and the very nature of the thing tested. We confirmed specificity of p53 family proteins (wtp53, GSTp53CD, GSTp63CD and GSTp73CD) by recognition of P1 sequence by all three chosen methods. In all setups tested sequence specific binding contributed distinguishably to protein-DNA interactions.

Acknowledgement

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P2 SAMPLE PREPARATION FOR SINGLE CELL ANALYSIS

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Summary
Development of sensitive, miniaturized and fast methods aims to reliable analyze even small amounts of samples. Micromanipulation systems such as laser capture microdissection enable to collect just single cells from cell suspensions or tissue samples. One option for further analysis is the bioluminescence reaction based on luciferin/luciferase conversion. This approach was used for detection of active caspases, caspase-3/7. Droplets based microfluidic systems provide benefits in biological, pharmaceutical and chemical research.

1 Introduction

Single cell analysis has become increasingly important for scientists working in basic or clinical research including hematology, stem cell biology, tissue engineering, immunology, developmental biology, neurology and cancer biology. It was assumed that cell populations are homogeneous, but the latest data show that heterogeneity exists even within a clonal population [1]. Therefore, assays performed in cell populations yield the average signal. Moreover, the cells of interest may be in the minority within the specimen. These discrepancies cannot be overcome using conventional biological techniques.

A number of single cell analysis methods have been established, e.g. microscopic imaging or flow cytometry. In addition, new microtechnology based tools have emerged in the last decade.

The purpose of our project is the development of instrumentation for manipulation with single cells and for chemiluminescent quantification of active molecules in various individual cells.

In the first step, the micromanipulation system is used for transfer of selected single cells. Photomultiplier tube (PMT) working in the photon counting regime allows for detection of the active molecules. This approach has been recently applied for caspase-3/7 detection in stem cells [2].

To further develop this technology, we have focused on laser capture microdissected (LCM) samples. We aim to employ a droplet based microfluidic system to analysis one single cell catapulted from cryopreserved tissue section.

Droplets based microfluidic systems provide numerous benefits in biological and chemical research [3]. Cell-containing droplets can be applied as a model for high-throughput chemical reactions, drug screening, toxicity testing as well as single cell analysis. This kind of microreactors can be manipulated and utilize in bio-testing. In this work, we present a designed platform for droplet generation and manipulation using dielectrophoresis (DEP) force [4].

The instrumentation and technology will be tested in monitoring of activation dynamics of caspases with focus on their novel functions in cellular differentiation.

2 Experimental design

2.1 Micromanipulation

Micromanipulation is a standard method for single cell handling. Typical micromanipulation systems consist of an inverted microscope plus a joy-stick operated, motorized micromanipulation platform. This system was used to collect single cells and insert these cells into the detection capillary (Fig. 1., 2.). The detection capillary was placed into the reflective chamber fixed by fine wire loop.

2.2 Detection of active caspase-3/7

The amount of active caspase-3/7 was detected in a photomultiplier tube based on bioluminescence reaction as described earlier [2].

Fig. 1. Micromanipulation system.

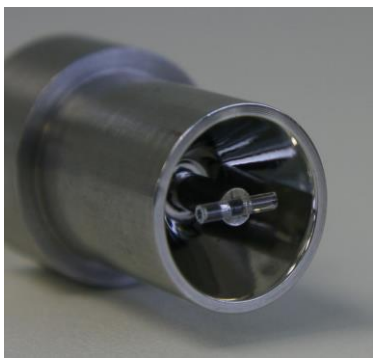

Fig. 2. Detection capillary placed inside the reflective chamber.

2.3 Laser capture microdissection

Laser capture microdissection enables to catapult cell clusters or single cells from cell suspensions or tissue sections. In our study tissue samples were frozen in liquid nitrogen and sliced by the Cryocut. Selected single cells were catapulted into the specially designed capillaries placed into the test tubes (Fig. 3.). The content of enzymes was quantified after the bioluminescence reaction.

Fig. 3. Capillary placed into the test tube.

2.4 Encapsulation of cells into droplets

Integrated microfluidic device with a DEP chip generates an emulsion which was collected onto the array and covered with an ITO-glass using parafilm "M" as a spacer (Fig. 4.). AC voltage 25V at frequency ranging from 50Hz to 1MHz across the electrodes was applied. Under these conditions, positive DEP of water droplets was observed. Droplets were attracted to the edges of the electrodes, where the gradient of the electric field is the highest.

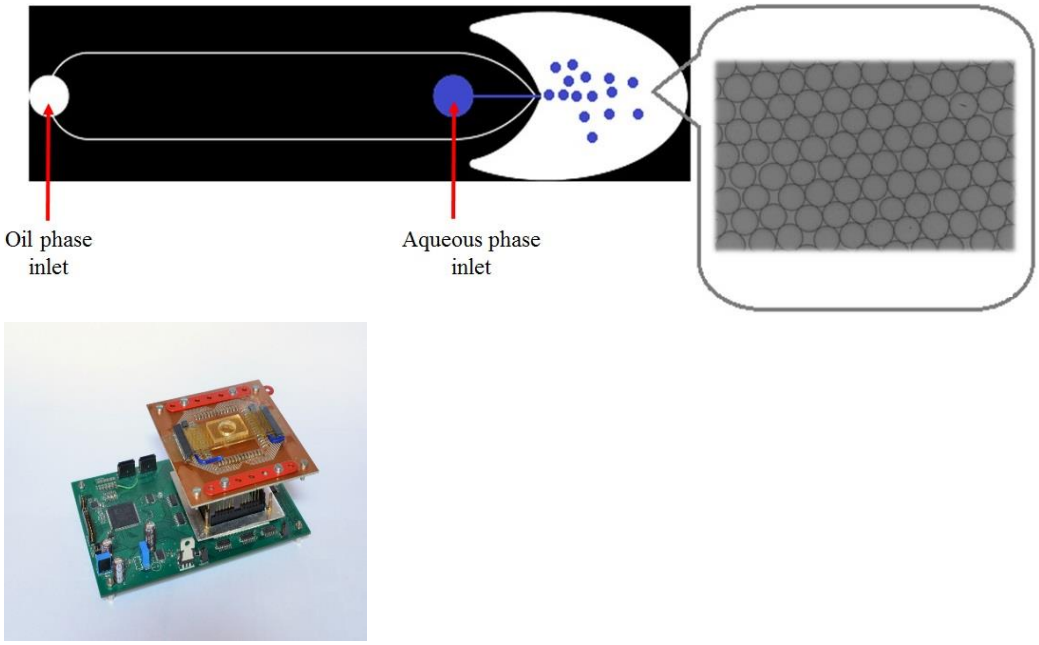

Fig. 4. Schematic picture of an integrated microfluidic device with a DEP chip. a) Flow focusing droplet generator, b) Top view of the microelectrode array connected to the generator.

3 Results and Discussion

The heterogeneous response of single cells towards identical stimulation is related to a discrepancy in the cellular signalling events of each cell [5]. This aspect cannot be covered by analyses using conventional biological techniques. Therefore, improved micromanipulation techniques have been developed in our department. While micromanipulation is a powerful and versatile method for capturing single cells, one of its drawbacks is the relatively long distance to transfer cells from a growth or storage medium into a tube for analysis. LCM enables one-step selection of one single cell even from histological sections into the special designed capillaries.

Regarding encapsulation, the manipulation flexibility can be further increased by using higher fields, thinner electrodes or different layout of the electrode array. Application of this designed platform for the future work involves single cell encapsulation and following detection by optical and mass spectrometric means.

Modern technologies of single cell analysis including high-resolution imaging methods open new possibilities for research in cellular biology and medical diagnostics.

4 Conclusions

The characterization and analysis of single cells is of increasing interest not only in biomedical research. Because the presence of various cell types in tissue samples complicates exact investigation of certain cells, different techniques have been developed to obtain single cells including micromanipulation, microdissection, and droplet microfluidic systems. Such miniaturized manipulation with tissue samples can provide improved sensitivity of further biological or chemical analyses.

Acknowledgement

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P3 LAYER-BY-LAYER CAPILLARY COATING FOR MODIFICATION OF ELECTROOSMOTIC FLOW IN CAPILLARY ELECTROPHORESIS

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Summary
In this work, we present a layer-by-layer approach for modification of capillary inner wall for capillary electrophoresis of biologically active compounds. The layer-by-layer coating was optimized using polybrene and polystyrenesulphonate. Further modification of the capillary by nanoparticles modified with polystyrenesulphonate was also evaluated.

1 Introduction

Capillary electrophoresis (CE) is known as a powerful analytical separation tool due to its high resolution, high efficiency, minimal sample volume, and ease of automation. Generally, CE separation is mostly performed in a fused-silica capillary and driven by the electroosmotic flow (EOF) [1]. Unfortunately, in the bare fused-silica capillary environment, because of the coulombic and hydrophobic interactions, many analytes (e.g. proteins) are adsorbed on the inner wall of capillary, which deteriorates the reproducibility and resolution [2]. These problems can be minimized by modification of the capillary wall [3].

Such surface modifications are achieved by permanent coating (a coating agent is covalently bonded to the capillary wall) and/or dynamic coating (interactions between a coating agent and the inner capillary wall can be hydrophobic, electrostatic, van der Waals and hydrogen-binding) [4]. Dynamic coatings have following advantages: (i) it is easy to generate those coatings under laboratory conditions; (ii) standard, cheap fused silica tubing can be used; (iii) the coating procedures are in most cases inexpensive; (iv) it is applicable over a wide range of buffer concentrations [5].

A number of agents can be used for dynamic modification of capillary wall, for example: polymers (cationic, anionic, non-ionic), amines (especially quaternary amines) or single chain and double chain surfactants (cationic, anionic, zwitterionic or non-ionic).

In the case of capillary dynamic coating, the modifiers are flushed through the capillary for a certain time and/or may be present in background electrolyte (BGE). Because the physical adsorption has a short lifetime and dynanic coating is less stable compared to permanently coated capillaries, the new method for dynamic coating was developed – successive multiple ionic-polymer layer (SMIL) coating. The coating is achieved by rinsing the oppositely charged ionic polymers solutions alternatingly, and reproducible coating could be achieved even though the capillary sources are

different. Besides of ionic polymers, we can also use other agents e.g. nanoparticles, which are known as the layer-by-layer (LBL) assembly. Assembly of layers can be monitoring by measuring of the electroosmotic flow (EOF), because capillary with negatively charged inner wall generate reversed EOF than the capillary with positively charged inner wall [1, 3].

In our experiments, we studied the use of layer-by-layer coating of the capillary for analysis of different biologically important compounds. First, we studied the performance of the coatings and then we separated model compounds using this coating.

2 Experimental

All experiments were performed by capillary electrophoresis HP ^{3D}CE with DAD detector (Agilent Technologies, Waldbronn, Germany) working at 200 nm. Untreated fused-silica capillary (MicroSolv Technology, Eatontown, NJ, USA) with a total length of 33 cm and effective length of 24.5 cm, an inner diameter of 50 μm was used. Applied separation voltage was set at +20 kV and -20 kV and the capillary cassette was thermostated at 25 °C. Injection of samples was provided by a pressure of 50 mbar for 5 seconds.

The capillary was flushed by 1.0 M NaOH for 20 minutes, then deionized water for 10 minutes and subsequently also with a buffer for 10 minutes before the first experiment. The coating procedure was performed by flushing the capillary alternatingly with solution of polybrene (PB), and solution of polystyrene sulphonate (PSS), each for 5 minutes. The capillary was flushed with a solution of 1.0 mM NaOH for 2 minutes, with deionized water for 2 minutes and with buffer for 4 minutes before each analysis. All rinsing was performed by pressure of 940 mbar. The buffer was prepared by dissolution of appropriate amount of phosphoric or boric acid in water and titration to desired pH by NaOH (50 %, m/m). Mesityloxide (MO), 0.01 % solution in methanol, was used as the EOF marker.

3 Results and Discussion

First, we studied the effect of layer-by-layer coating approach on magnitude and direction of the electroosmotic flow. After washing of the each layer, we measured the EOF by MO as the EOF marker. The results are shown in Fig. 1. It is clear that the system polybrene (PB) – polystyrenesulphonate (PSS) is suitable for layer-by-layer type of the coating. Moreover, when the PSS is used as the final layer, the magnitude of EOF is lower than that when using PB as the final layer.

Then, the stability of the coating was tested under different pH. A set of phosphate and borate buffers at pH ranging from 2.0 to 10.0 was used. Good stability (RSD of the EO mobility values were lower than 1 %) was observed in the pH region 2.5 to 8.0 in 5 successive runs. Finally, overall stability of the coating was tested using 50 successive runs in pH 7.5. In this case, the RSD of the EO mobility was lower than 3 %. Finally, as the pilot testing, a coating by PB and polystyrenesulfonate-modified silver nanoparticles were studied. Here, we observed similar promising behavior as with the PB-PSS system.

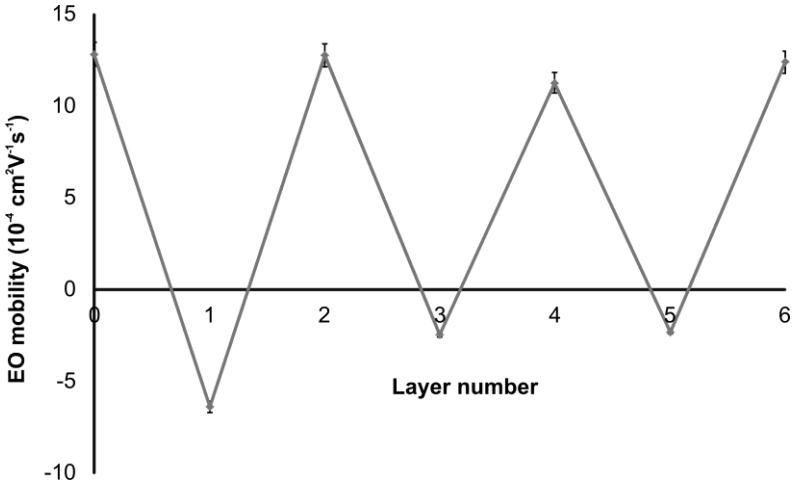

Fig. 1. The effect of layers on the EO mobility.

4 Conclusions

In this work, we presented a layer-by-layer approach for modification of capillary inner wall for capillary electrophoresis. The layer-by-layer coating was optimized using polybrene and polystyrenesulphonate. Further modification of the capillary by nanoparticles modified with polystyrenesulphonate was also evaluated. This coating procedure provides very interesting pilot results that can be used for analysis of biologically active compounds.

Acknowledgement

The financial support of the research by the Ministry of Education, Youth, and Sports of the Czech Republic (project NPU LO 1305), the Grant Agency of the Czech Republic (P206/12/1150), and the Student project UP Olomouc IGA_PrF_2014_031 is gratefully acknowledged.

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P4 CITP ANION ANALYSIS OF BEVERAGES

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Summary
The new analytical method for the determination of content of sulphates and sulphites in the presence of carbonates by means of capillary isotachophoresis (CITP) was developed and this procedure was utilized for the analysis of wines, beers and non-sparkling mineral water samples. In case of beer and wine samples, the content of organic acids (e.g. benzoic, citric, lactic, acetic and sorbic acids) was determined in order to evaluate their impact on results obtained by developed technique.

1 Introduction

Sulphites and sulphates are widely used in food industry, especially in the production of some alcoholic drinks (e.g. beer, wine). Sulphites are a common antifungal agent, whereas sulphates are primarily used to support the fermentation of beer and drinking water treatment. Organic acids are added in beer and wine as preservatives and flavour enhancers. Although these substances are recognized by legislation as approved food additives, it is suitable to check their contents. Sulphates and sulphites as well as organic acids in foods are not dangerous in small amount; on the other side they may have side effects on the human body such as headache, allergic reactions, nausea or vomiting [1]. This contribution describes CITP of beverages since it is suitable as fast method for qualitative and quantitative analysis of samples having small volumes.

2 Experimental

Analysis was carried out on the electrophoretic analyser EA 102 (Villa Labeco, Spisska Nova Ves, Slovakia). The length of the PTFE tube was 140 mm and a diameter was 0.3 mm. In this work, three working systems [2-4] were used for analysis of different samples (see Table 1). Wine, fruit beer and non-sparkling water samples of Czech origin were analysed since they contain analytes in broad concentration region.

All samples were diluted (5-10) times prior analysis and carbon dioxide was also removed in ultrasonic bath in some cases. The operational systems (see Table 1) were used for CITP analysis of chosen analytes.

Table 1. Operational systems employed in ITP analysis.

Parameters	Anionic analysis		
System Nr.	1 (sulphates)	2 (sulphites, carbonates)	3 (organic acids)
Leading Electrolyte	10 mmol/L Cl^-	10 mmol/L Cl^-	10 mmol/L Cl^-
Terminating Electrolyte	10 mmol/L Citric acid	5 mmol/L His + 5 mmol/L Tris	5 mmol/L MES
Counter Ion	10 mmol/L β-alanin + 3 mmol/L BTP	Tris	5.5 mmol/L BTP
pH	3.6	7.7	6.2
Additive		0.1% HEC	

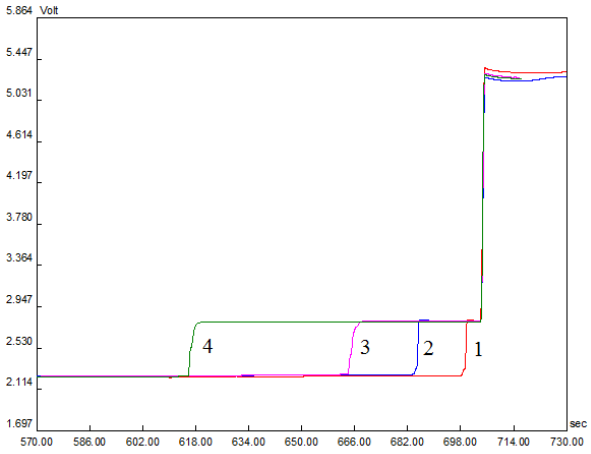

Fig. 1. Determination of sulphates in Rajec sample: 1 – 0, 2 – 10 mg.l-1, 3 – 25 mg.l-1, 4 – 50 mg.l-1.

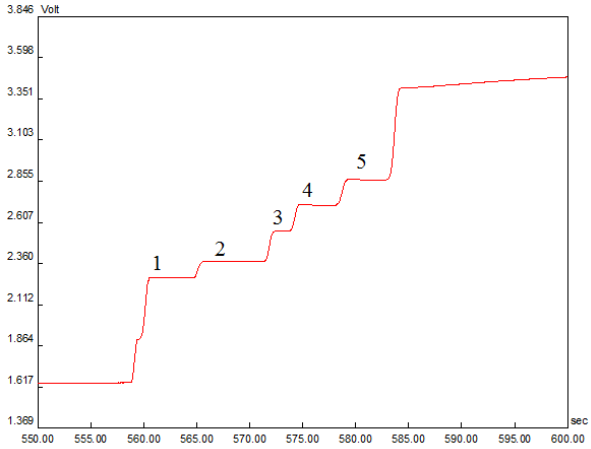

Fig. 2. Model sample of organic acids (RSH): 1 – citric acid (0,308), 2 – acetic acid (0,360), 3 – lactic acid (0,440), 4 – benzoic acid (0,564), 5 – sorbic acid (0,640).

3 Results and discussion

The mentioned beverages were analysed by CITP method as described above. Some isotachophoregrams are given in Figs 1 and 2. Results of non-sparkling mineral water samples were compared with the declared values. The carbonate content was slightly higher as well as values for sulphates. Small amount of sulphites was detected in all three water samples however their content was not declared. The amount of free sulphites in all wine samples was lower than 150 $mg.l^{-1}$ while the Czech legislation limit is set to be lower than 210 $mg.l^{-1}$. Content of carbonates was about 200 $mg.l^{-1}$ and the amount of sulphates in all wine samples was detected at low concentration. Beer samples were analyzed and the concentration of selected organic acids (e.g. benzoic, citric, lactic, acetic and sorbic acids) was determined. Acetic acid was not detected in all analyzed samples while the content of citric acid was estimated in range 2000-11220 $mg.l^{-1}$, concentration of benzoic and sorbic acids in range 1000-2000 $mg.l^{-1}$ and lactic acid was found in the range of 3000-6000 $mg.l^{-1}$.

4 Conclusion

This contribution presents the possibility of CITP technique for anion analysis of three types of beverages. The chosen analytes (e.g. sulphates, sulphites, carbonates, and organic acids) were determined in samples of wine and beer of Czech origin. In addition, samples of non-sparkling mineral waters were analysed for the concentration of sulphates and sulphites in the presence of carbonates. Results for non-sparkling mineral water samples were compared with declared values.

Acknowledgements

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P5 THE DETERGENT EFFECT DURING N-GLYCAN RELEASE FROM STANDARD GLYCOPROTEINS

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Summary
The N-glycan release from glycoproteins with Peptide-N-Glycosidase (PNGase F) enzyme is a time-consuming process, and the reproducibility is dependent on the unfolding rate, that may cause difficulties in reproducibility during the study of unknown glycoproteins. In this study the effects of two nonionic detergents were investigated in the enzymatic deglycosylation process.

1 Introduction

Analytical glycomics is a newly emerging scientific discipline aiming comprehensive characterization of a large number of glycans. The glycome is the entire complement of sugars, whether free or in complex molecules in an organism. The study of carbohydrate moieties is in the recent focus of interest, because they take part in several significant processes such as cell-cell recognition, cell development and differentiation, the interaction between cells and hormons, antibodies, etc. [1] Analytical glycomics opens up new avenues for biomarker discovery, as glycan structures are highly variable and change in in pathological conditions. [2] Therefore, understanding the glycosylation of proteins is important that requires high resolution and sensitive bioanalytical methods.

Among the available analytical techniques, capillary electrophoresis (CE) has proven to be one of the most efficient ones for the analysis of glycoproteins, oligosaccharide profiling and sequencing with the help or exoglycosydase digestion techniques [3]. From the practical point of view, only a small amount of sample is needed for CE analysis to provide high resolution separation with good reproducibilities. [4] In this presentation we show that the both the amount of the released N-glycans and the reproducibility of release are enhanced with the use of the appropriate non-ionic detergents.

2 Experimental

Ribonuclease B (Sigma-Aldrich, St Louis, MO) was digested with Peptide-N-Glycosidase F (Prozyme, Hayward, CA) enzyme overnight at 37 °C. The digestion process was evaluated in five different buffer systems containing various types and amounts of non-ionic detergents: 1) PBS buffer (pH=7.4); 2) 0.6% NP-40 non-ionic detergent in PBS (pH=7.4); 3) 1.3% NP-40 in PBS (pH=7.4); 4) 2.0% NP-40 in PBS (pH=7.4) and 5) 2.0% Triton X-100 non-ionic detergent in PBS (pH=7.4). After the digestion process, the remaining polypeptide chains were precipitated with ice-cold

ethanol, centrifuged and the released glycan-containing supernatant was dried in a centrifugal vacuum evaporator. The dried sugars were labeled with 8-aminopyrene-1,3,6-trisulfonic acid, trisodium salt (APTS) fluorescent dye (Beckman-Coulter, Brea, CA) via a reductive amination reaction. The labeled samples were analyzed by a P/ACE MDQ (Beckman-Coulter) capillary electrophoresis system with laser induced fluorescence (CE-LIF) detection (488 nm excitation / 520 nm emission) using the N-CHO kit (Beckman-Coulter).

3 Results and Discussion

Ribonuclease B was PNGase F digested in five different buffer systems in order to investigate the release efficiency and reproducibility of process. All five buffer systems were based on PBS buffer (pH=7.4), but differed in the detergent content: A set: PBS buffer without detergent; B set: 0.6% NP-40 in PBS; C set: 1.3% NP-40 in PBS; D set: 2.0% NP-40 in PBS; E set: 2.0% Triton X-100 in PBS.

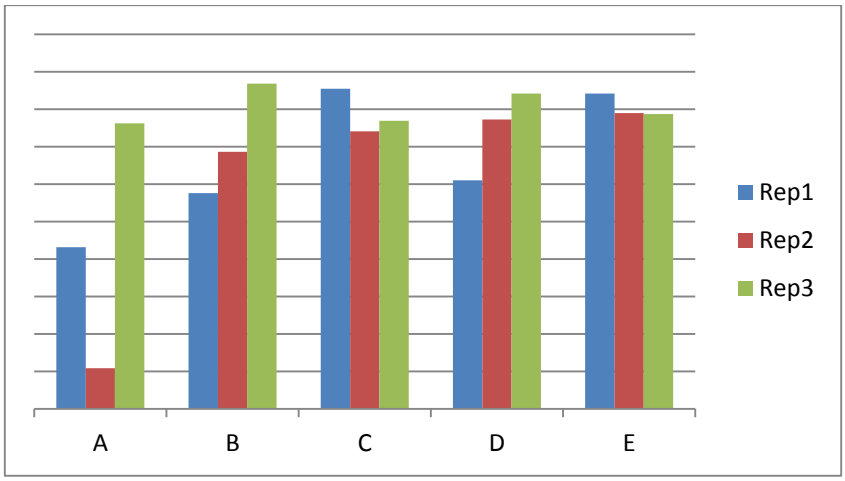

Fig. 1. Reproducibility of Man5 release from Ribonuclease B using five different buffer systems as listed in the Experimental section.

Figure 1 shows the reproducibility of glycan release in the different non-ionic detergent containing buffer systems. The peak area reproducibility of the digestion process in plain PBS buffer was quite low (A set of bars). Bar sets from B to E show the quantitative results of the deglycosylation process in the presence of two non-ionic detergents revealing improved efficiency and reproducibility.

Table 1 depicts the peak areas and their relative standard deviation from the different deglycosylation conditions. Without the use of any detergents, the relative standard deviation of Man5 and Man6 peak areas were 75%. The addition of detergents significantly improved these values. The addition of 2.0% TritonX-100 detergent provided the highest reproducibility and enhanced the effectiveness of the procedure. Moreover, corresponding peak areas did not change using this detergent type.

Table 1. Peak areas and their relative standard deviation (Man5 and Man6) under different deglycosylation conditions.

	Peaks	Rep1	Rep2	Rep3	Average	RSD
PBS	1. Man5	8634820	2167210	15244008	8682013	75
	2. Man6	6848760	1703799	11922033	6824864	75
	Peak rate (%)	1,26	1,27	1,28		
0.6% NP-40	1. Man5	11517281	13734771	17363255	14205102	21
	2. Man6	8960464	10709140	13619221	11096275	21
	Peak rate (%)	1,29	1,28	1,27		
1.3% NP-40	1. Man5	17100973	14831304	15389132	15773803	7
	2. Man6	13569378	11630404	12006964	12402249	8
	Peak rate (%)	1,26	1,28	1,28		
2.0% NP-40	1. Man5	12198379	15462586	16837398	14832788	16
	2. Man6	9502595	12191860	13236407	11643621	17
	Peak rate (%)	1,28	1,27	1,27		
2.0% TritonX-100	1. Man5	16837398	15801820	15743858	16127692	4
	2. Man6	13236407	12502059	12596609	12778358	3
	Peak rate (%)	1,27	1,26	1,25		

4 Conclusions

The effect of non-ionic detergents in PNGase F deglycosylation process was investigated. With the use of NP-40 and TritonX-100, the latter one resulted in improved deglycosylation efficiency, greater amount of released glycans and enhanced reproducibility.

Acknowledgement

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P6 SIMULTANEOUS ANALYSIS OF DEXRAZOXANE AND ITS PUTATIVE ACTIVE METABOLITE USING HPLC-MS/MS

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1 Introduction

Anthracyclines are effective anticancer drugs; however their use is complicated by their serious cardotoxicity. Bisdioxopiperazine drug dexrazoxane (DEX, Fig. 1) is currently the only clinically used drug to reduce an anthracycline induced heart injury. However its mechanism of action still remains elusive. The generally accepted theory suggests that the metabolite ADR-925 (Fig. 1) formed by an enzymatic bioactivation of DEX inside cardiomyocytes is responsible for the protecting effect thanks to its iron chelation properties.

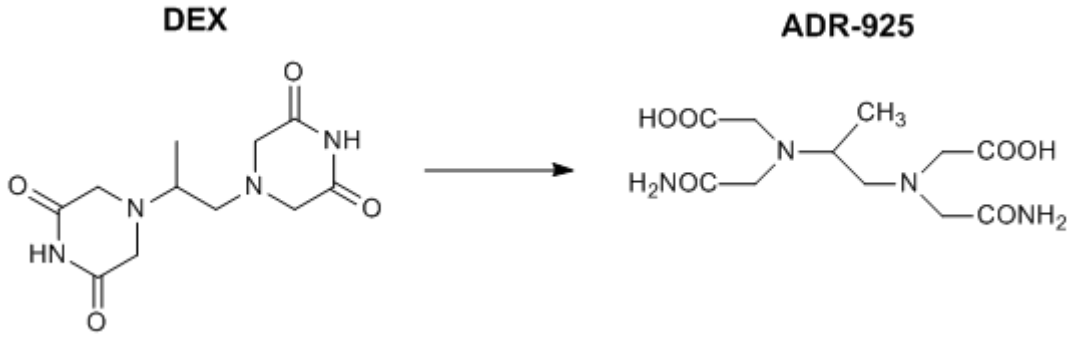

Fig. 1. Conversion of DEX to ADR-925.

On the other hand, recent evidence suggests that the main role might be carried by the inhibition of topoisomerase II by parent DEX [1]. A limited knowledge regarding the exact role of the metabolite ADR-925 and parent DEX in cardioprotection is associated with their complicated simultaneous analysis in a relevant biological material. Although our first developed HPLC-MS method for simultaneous analysis of DEX and ADR-925 in biological samples was successfully applied to a pharmacokinetic study, it suffers from several drawback, where the most relevant is the long analysis time (30 min/run). Therefore the aim of this study was to simplify the overall analytical conditions and transfer the method to the triple quadrupole mass spectrometer to provide a suitable analytical tool for processing the large numbers of biological samples in reasonable time.

2 Experimental

The previously developed HPLC-MS method employed the LCQ Advantage MAX ion trap mass spectrometer (Thermo scientific) and chromatographic column Synergi Polar-RP (3x150 mm, 4 µm) protected with a guard column. The mobile phase was composed of 2 mM ammonium formate (AMFM) and methanol in a gradient mode. 1,2-diaminopropane-N,N,N′,N′-tetraacetic acid and 1,2-bis(3,5-dioxopiperazin-1-yl)ethane was used as an internal standard for ADR-925 and DEX, respectively.

New method utilized LCMS-8030 triple quadrupole (Shimadzu) and chromatographic column Zorbax Bonus-RP (3x150 mm, 3.5 µm) protected by a guard column. Mobile phase was composed of 0.05% HCOOH, methanol and acetonitrile in a gradient mode. 4,4'-(propane-1,2-diyl)bis(1-methylpiperazine-2,6-dione) was used as an internal standard for both ADR-925 and DEX.

All plasma samples were treated by precipitation with 90% methanol, centrifuged (16,800 g, 10 minutes) and filtered using 0.22 µm filters.

3 Results and discussion

The original HPLC-MS method for simultaneous analysis of DEX and ADR-925 took 30 minutes, which made it too much time consuming for routine sample analysis. Retention times of 5.1 and 20.9 minutes were acquired for ADR-925 and DEX, respectively. This method was validated according to FDA guideline with LLOQ 300 ng/ml in spiked plasma samples.

C18, biphenyl, phenyl alkyl ether phases tested as a stationary phases for new HPLC-MS/MS method provided only poor retention of highly hydrophilic ADR-925. The best results were obtained using phase with an embedded amide linkage in the C14-alkyl chain – namely Zorbax Bonus-RP (3x150 mm, 3.5 µm). Also various mobile phases consisting of methanol, acetonitrile, water and AMFM buffers were tested. Based on optimization, the final mobile phase was composed of 0.05% HCOOH, methanol and acetonitrile in gradient mode. Retention times of 4.5 and 5.2 minutes were acquired for ADR-925 and DEX, respectively. Besides the simplification of mobile phase composition only one internal standard was necessary because of much closer retention times of ADR-925 and DEX compared to previous method.

Methanol, acetonitrile and their mixtures with water were tested for plasma precipitation in different ratios. Plasma samples were finally precipitated by 90% methanol, which increased recovery of ADR-925 compared to 100% methanol because of its hydrophilic properties. Its amount was optimized to 1:3 ratio to plasma with respect to compromise between maximum possible method sensitivity and sufficient protein removal. Samples were centrifuged and possible remaining proteins were removed by filtration through 0.22 µm filters.

Analysis time was shortened to only 10 minutes, which is three times better result compared to the previous method. In addition, method sensitivity was increased significantly. LLOQ of the method reached 100 ng/ml in spiked plasma samples, which is one third of the previous method. On the other hand, as for the previous method, the column needed to be flushed with the 2 mM EDTA solution to solve low

sensitivity and reproducibility of the method at low concentrations of ADR-925, which is probably connected with its strong chelation properties.

4 Conclusions

Novel HPLC-QqQ method capable to assay ADR-925 along with DEX in plasma was developed. Composition of mobile phase was simplified compared to previous method and also number of required internal standards was reduced. The most importantly, time required for simultaneous analysis of both compounds was shortened three times and sensitivity of the method was significantly increased as well. This newly developed method provides significantly improved parameters and thus allows analyzing of high numbers of biological samples in acceptable time and with sufficient sensitivity. After full validation this method will be utilized for analysis of biological samples from advanced *in vivo* experiments focused on investigation of the real importance of ADR-925 in cardioprotection of DEX.

Acknowledgement

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P7 SPECTROPHOTOMETRIC DETERMINATION OF METALLOTHIONEINS IN FISH

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1 Introduction

Metallothioneins (MTs) are a low molecular weight intracellular proteins (metal binding proteins, MBP) with a high cysteine content. The thiol groups (-SH) of cysteine are able to bind essential or toxic metal ions, such as copper (Cu,) zinc (Zn) as essential, cadmium (Cd), mercury (Hg) and some others as toxic. This binding reduces the toxic effect of free cytosolic metals on proteins and metabolic function of enzymes [1, 2]. The biological function of MTs is the important role in homeostatic

controle, metabolism of essential metals and is involved to detoxification of excess amounts of both essential and non-essential trace metals. Due to this role in detoxification of heavy metals MTs are assumed to be acute phase proteins of sudden increase of high intracellular metal concentration [2, 3]. Since their discovery as cadmium binding proteins from horse kidney in 1957 [4] MTs are continuously studied [5]. Studies of their role in detoxification of heavy metals in fish presented in previously studies [6] were focused on fish exposed to polluted aquatic environment. It is generally known that fish can accumulate Cd, Cu, Zn in tissues, especially in gills, liver and kidney [7]. MTs bind Zn, but in excess of Cd or Cu, zinc can be easily replaced by these metals [8]. MTs rich cells are resistant to toxic effect of cadmium more than cells that cannot synthesize MTs, as published in experimental study [9]. Structure of MTs is important for their function. MTs molecules are formed by two binding domains composed of cysteine clusters – Fig. 1. Covalent binding of metal atoms involves sulfhydryl cysteine residues [10].

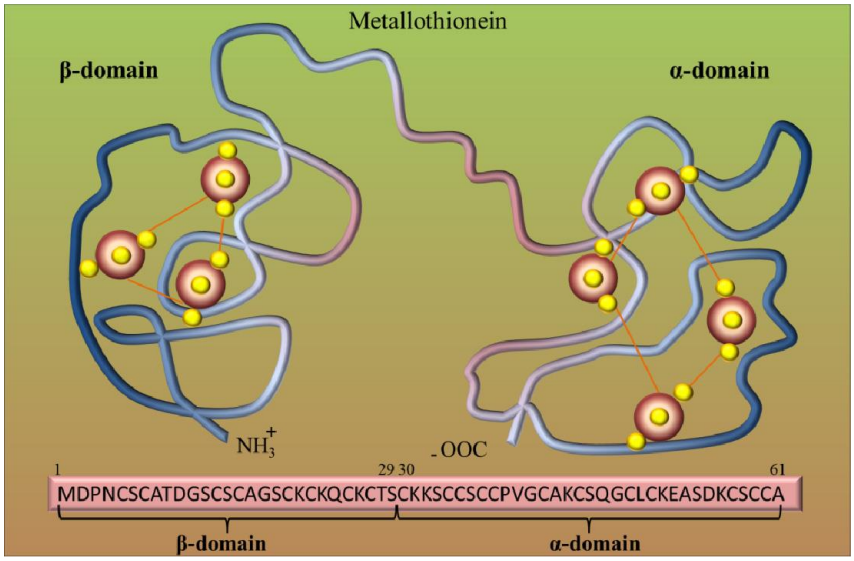

Fig. 1. Metallothionein´s structure. Model of two binding sites of metallothionein. Red small disc is metal atom (Zn, Cd, etc.) and small yellow points are sulfur atoms. Adopted from [10].

In fish, elevated concentration of cadmium in the water or in the diet leads to induction of MTs synthesis in tissues [11]. MTs synthesis in fish can be induced by endogenous factors, too. Some parameters of water, such as alkalinity and acidity or age of fish can evaluate accumulation of cadmium or synthesis of MTs in tissues [12]. MTs are important in detoxification and regulation of metals homeostasis and can evaluate health status of fish.

Various methods to determine of MTs in animal tissue can be used. Classical techniques for the determination of MTs are polarography, immunological assays as radioimmunoassay, enzyme-linked immunosorbent assay [1]. Brdicka´s reaction is frequently used for the electrochemical detection of MTs [13]. Next techniques from recent period prefer methods as liquid chromatography, gas chromatography or

capillary zone electrophoresis or hybrid techniques based on a combination of separation techniques with selective detectors [1]. Disadvantage of these analytical methods is the use specialized laboratory equipment.

The aim of our study was prepare and use easy suitable method for quantification of MTs in fish tissue as the biomarker of water pollution and contamination of fish organism by toxic metals.

2 Experimental

The principle of method to evaluation of MTs in tissue samples homogenate is based on photometric reaction, chemical determination of cysteine residues by the Ellman´s reaction [14, 15, 16]. Endogenous thiols such as glutathione, free cysteine, proteins etc. do not interfere with the reagents of this method. MTs are prone to oxidation during the procedure. Preparation of tissue homogenate requires teflon-glass tissue homogenizer. After homogenization, metallothioneins are alcohol precipitated, than resuspended in highly denaturating buffer. During preparation of samples homogenate and extraction steps metallothioneins are protected against proteases and oxidation by proteases inhibitor, the component of extraction buffer. After reaction with the Ellman´s reagent Bis - (3-carboxy-4-nitrophenyl) disulfide form colored complexes. The intensity of colored samples is measured e.g. on UV-VIS spectrophotometer Cintra, the wavelength $\lambda = 412$ nm.

3 Results and Discussion

This method were used and optimized for determination of MTs in tissue samples (liver and muscle) obtained from silver crucian carp (*Carassius gibelio*). Samples were homogenized in ice-water bath by Teflon-glass grinder. We prepared and measured 5 concentrations of standard solution (4 mM reduced glutathione) for construct standard calibration curve. The relative amount of MTs could be re-calculated against number of cysteine molecules in species (fish) - Fig. 2. The data from analysis of tissue are usually expressed as nmol thiol/mL and amount is based to total amount of proteins in sample, thus nmol thiols/mg proteins or nmol thiols/g tissue.

MTs in fish are quick and clear biomarker of heavy metal pollution and heavy metal exposure, but so marker of MTs formation due to a long-term existing normal realistic aquatic environment concentration of metals [2, 3, 12].

The method is sufficiently sensitive for determination concentration of MTs in fish. The limit of detection has been less than 200 pico moles of metallothionein. We are not limited by the amount of sample for use in fish tissue. MTs are determined in small part of tissue or body sample about 0,1 grams, may be less, depending on the amount of MTs in the sample.

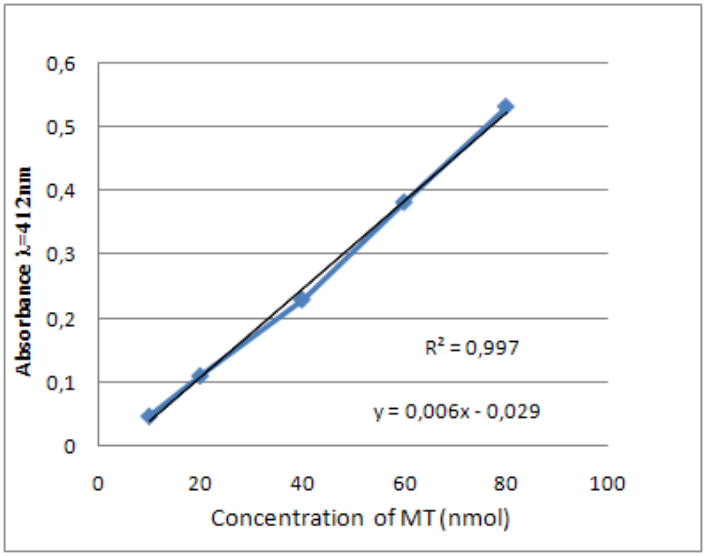

Fig. 2. Calibration curve of MT.

4 Conclusions

This spectrophotometric method is available to laboratories for routine analysis of MTs as a biomarker of heavy metal pollution and heavy metal exposure and MT formation due to a long-term existing normal realistic aquatic environment concentration of metals.

Acknowledgement

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P8 MONITORING OF SELECTED PARAMETERS IN AMELANCHIER ALNIFOLIA EXTRACTS BY UV-VIS-NIR AND EPR SPECTROSCOPY

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Summary
This study focuses on characterization of Saskatoon berries extracts using spectroscopic techniques. Cultivars of *Amelanchier alnifolia* were extracted in three different solvents (distilled water, 50% ethanol and DMSO) and these extracts were tested by UV-VIS-NIR and EPR spectroscopy. Following parameters were investigated: total polyphenolic content, total anthocyanins content, color characteristics, ferric reducing antioxidant potential and total antioxidant activity using $^{\bullet}$DPPH and ABTS$^{\bullet+}$assays.

1 Introduction

Saskatoon berry (*Amelanchier alnifolia)* is a deciduous scrub or small tree which belongs to the rose family (*Rosaceae)*. It comes from North America where it has been used for centuries for therapeutical, pharmaceutical and food purposes. It has sweet red or dark-purple pomes, which are a valuable source of chemical substances with an antioxidant effect, especially phenolic acids, flavonoids, anthocyanins and vitamins [1]. These compounds are responsible for its health benefits, including

antimutagenic and anticarcinogenic activity, for the prevention of various cancers and age-related diseases [2].

The aim of this study was to monitor selected physico-chemical and nutritional parameters of *Amelanchier alnifolia* cultivars using spectroscopic techniques with emphasis on the effect of solvents on the monitored parameters. The differences among varieties and the year of production were assessed. For this purpose, selected multivariate statistical methods were used.

2 Experimental

2.1 Examined samples

Five cultivars of Saskatoon berries: Lamarckii Balerina (AL), Thiessen (AT), Ostravsky (AO), Tišnovsky velkoplody (AV), Tišnovsky školsky (AS) were analysed. Fruits were harvested in an experimental gene-fund orchard of Mendel University in Brno (Zabcice, CZ) by experienced agronomists during the period 2012−2013. Then immediately frozen at −18°C and stored until analysis.

2.2 Preparation of extracts

Three different types of extracts were prepared (in distilled water, 50% ethanol and dimethylsulfoxide (DMSO). The extract procedure is described in work of Butorova [3]. Two extracts of every cultivar were prepared, every sample was analysed two times (number of experiments, 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 was determined applying Folin-Ciocalteau modified method [4]. Total anthocyanins content (TAC) was determined by pH differential method [5]. Colour characteristic (colour coordinates L*, a* and b*, chromaticity and hue angle) of extracts were performed directly from measured spectra by means of ColourLite Panorama Shimadzu software (LabCognition Analytical Software, Germany) using the D65 day light illuminant and 10° standard observe angle. The capability of Saskatoon extracts to reduce Fe^{3+} to Fe^{2+} was tested according to method of Chyau et al. [6].

2.4 EPR experiments

All experiments were performed using a portable X-band EPR spectrometer e-scan (Bruker, Germany) with accessories. $^{\bullet}$DPPH and $ABTS^{\bullet+}$ radical-scavenging activity assays were prepared on this way: 300 µl of sample was mixed either with 700 µl of $^{\bullet}$DPPH solution in ethanol or the solution of $ABTS^{\bullet+}$ in water (initial concentration c=0.1 mmol/l). The mixture was purged with 2 ml of air and immediately transferred into the EPR flat cell. EPR measurements started exactly 3 min. after $^{\bullet}$DPPH or $ABTS^{\bullet+}$ addition and a set of 10 EPR spectra was recorded in time domain during 15 min.

3 Results and Discussion

The antioxidant activity of extracts was measured using various methods, only selected parameters are presented in this paper. Results of TAC confirm that Saskatoon berry is a good source of anthocyanins. Usually the content of anthocyanins in Saskatoon berry ranges from 251 to 1790 mg/kg [1, 7] for methanol extracts. Experimentally observed levels of TAC in ethanol extracts range from 99.3 to 920.4 mg/kg, in aqueous extracts range from 4.2 to 88.7 mg/kg and in DMSO extracts range from 362.2 to 1 178.5 mg/kg. Lower values of TAC in aqueous and some ethanol samples may be related to the nature of the solvent, location of growth, climatic conditions and manipulation with fruits [8].

The highest concentration of anthocyanins was determined in the variety Ostravsky 2012, while the lowest in the variety Thiessen 2013. The results of TAC indicate seasonal effects – regardless on the variety, the year 2012 was characterized by higher content of TAC, in comparison with the year 2013; in case of ethanol and aqueous extracts these differences are statistically significant (P_{EtOH}=0.0378, P_{H2O}= 0.0080). TAC is highly influenced by choice of extractant. TAC decreases in the direction of DMSO>50% ethanol>distilled water. With respect to the values of relative permittivity of solvents, the observed differences between agents can be related to different polarity of solvents. Based on the results we can conclude that DMSO was the most appropriate solvent for the extraction of these compounds.

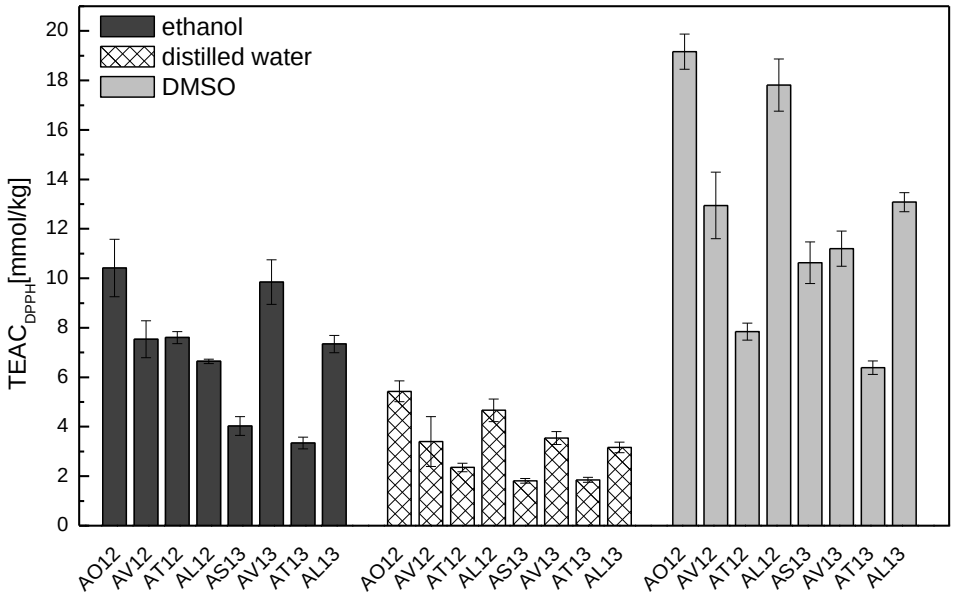

Fig. 1. The average values of TEAC•$_{DPPH}$ (mmol/kg) determined in ethanol, aqueous and DMSO extracts of Saskatoon berries. Specification of samples - see chapter 2.1.

The average values of total antioxidant activity expressed as the TEAC•$_{DPPH}$ are shown in Fig. 1. As with the determination of total anthocyanins, the trend is retained, that solvent influences TEAC•$_{DPPH}$ values. It is evident that the radical-scavenging activity of extracts depends on the variety and the year of production. In accordance with the above mentioned results, the year 2012 was characterized by

higher values of $TEAC_{\bullet DPPH}$ than the year 2013 and the highest antioxidant activity was determined in the variety Ostravsky 2012, the lowest in the variety Thiessen 2013. The results also correlate well with the observation in the case of $ABTS^{\bullet+}$ test and are in good agreement with quantified concentrations of anthocyanins; this fact was also confirmed by multivariate statistical methods.

4 Conclusions

Three extraction systems were tested in order to determine which one is the most suitable for the isolation of functional substances from Saskatoon berries. Dimethylsulfoxide seems to be the most suitable solvent for all parameters, but from the food point of view it is not suitable due to its flammability and irritation effect. Extracts in 50% ethanol and/or distilled water are rather interesting. This study also shows that Saskatoon berry cultivars grown in Czech Republic have a high radical scavenging activity, which is attributed to the presence of polyphenolic compounds, mainly anthocyanins.

Acknowledgement

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P9 EFFECT OF SLEEVE GASTRECTOMY ON PARAMETERS OF BONE METABOLISM AFTER RADICAL WEIGHT LOSS

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1 Introduction

In recent years, a number of signal molecules were discovered produced by adipose tissue, whose physiological significance exceeds the general metabolic aspects of the organism [1]. Therefore, fat is currently regarded as an endocrine organ, whose hormones modulate the function of many systems including the skeleton [2]. These hormones also include adipokines that modulate bone metabolism both at the tissue level (leptin, adiponectin) and indirectly – by activating neuro-humoral hypothalamic centers – leptin [3]. Moreover, leptin stimulates osteoprotegerin as an inhibitor of RANK ligand at a decrease in bone resorption [4, 5]. On the other hand, it is becoming apparent that there is a negative correlation between adiponectin and bone density. An important molecule within the relation between energy metabolism and bone is Ghrelin. Although the mechanism of effect of ghrelin on the skeleton is not exactly known, some clinical studies report a positive correlation between circulating ghrelin and bone density [6].

2 Experimental

The study included 40 women on the basis of predetermined criteria (the diagnosis of morbid obesity according to the guidelines IFSO, a person with BMI> 40 kg/m^2 or a BMI> 35 kg/m^2 with associated comorbidities, persons aged 20 or over and their consent to cooperate). The probands in the study underwent laparoscopic sleeve gastrectomy. Six and twelve months following the surgery, additional serological measures were taken. To reduce analytical variation, hormones and cytokines from all patients were analyzed in the same run. Blood samples were kept at -80 ° C until the time of analysis. Serum levels of ghrelin, leptin, adiponectin, osteoprotegerin and sRANKL were analyzed by ELISA assay (Biovendor - Laboratory Medicine, Czech Republic). Serum level of parathyroid hormone was assessed by Immulite 2500. Serum levels of P1NP, osteocalcin and CTx were assessed by Roche. All the statistical tests were evaluated at the significance level of 5 %. The Shapiro-Wilk test was used to test normality of the data. The effects of gender and intragastric balloon in the full model were tested using the F-test. The t-statistic was used to test if each particular coefficient of explanatory variables was equal to zero. These analyses were performed using R software.

3 Results and discussion

Twelve months after the surgery, we observed a significant decrease in total weight and content of adipose tissue. The summary of the results of the dynamics of change are presented in Table 1.

Table 1. Overview of anthropometric measured parameters.

N = 40	Baseline	6 months	12 months	P - value
Weight [kg]	116,4 ± 18,4	90,5 ± 17,3	85,6 ± 16,4	0,001
BMI [kg/m^2]	42,4 ± 5,2	32,9 ± 4,9	30,9 ± 4,9	0,001
Fat [%]	45,4 ± 6,5	36,0 ± 6,9	33,4 ± 7,2	0,001
Lean body mass [kg]	63,2 ± 7,0	57,08 ± 7,2	56,2 ± 7,7	0,001

Data are expressed as mean ± SD. P-values refer to significantly different values between baseline and 6 months following the surgery (F-test).

In the course of monitoring through 12-months, significant changes occurred in serum concentrations of osteocalcin (OC), P1NP (N-terminal procollagen type 1), CTx (C-terminal telopeptide of type I collagen). Changes in the concentration of osteoprotegerin (OPG) were not significant after 3 months, but subsequently, after 6 months, there was a statistically significant decrease in serum concentration. There was also a decrease in serum concentrations of PTH (parathyroid hormone), but the changes were not statistically significant. Over the first 6 months, sRANKL was significantly decreased, after 12 months, there has been a rise in serum concentrations. However, elevated sRANKL was not significant from a statistical point of view. The summary of the results of the dynamics of change are presented in Table 2.

Targeted weight loss leads to bone loss in both young [7] and elderly women [8]. The results of prospective studies show that women with a lower BMI lose more bone mass after menopause than women with higher BMI [9]. There are several effects of obesity (adipose tissue) on the skeleton. The skeleton adapts to increased mechanical load (tension and compression stimulates osteoblastic activity and reconstruction of bone trabeculae). Adipose tissue provides some mechanical protection of bone in case of a fall. Adipose tissue does not have only mechanical effect; at present, it is a generally accepted fact that adipose tissue produces a number of hormones and cytokines, thus being very actively involved in the regulation of food intake, a number of metabolic processes including bone metabolism of bone tissue [10].

Table 2. Dynamics of changes in adipose tissue hormone serum and osteomarker levels at a check-in 6 and 12 months after the surgery.

N = 40	Baseline		6 months	12 months	P-value
Adiponectin	15.7 ± 6.7		14.61 ± 5.4	29.75 ± 15.0	0.000
Leptin	46.21 ± 8.6		28.48 ± 15.5	22.96 ± 9.1	0.007
CTx	0.21 ± 0.1		0.433 ± 0.2	0.434 ± 0.3	0.012
PTH	4.01 ± 1.8		3.92 ± 2.1	3.45 ± 1.7	NS
sRANKL	256.74 ± 170.8		157.38 ± 98.2	227.06 ± 166.3	NS
OPG	4.26 ± 1.4		3.88 ± 1.0	2.79 ± 1.4	0.017
P1NP	32.54 ± 11.0		47.51 ± 20.1	54.83 ± 23.5	0.003
Osteokalcin	13.99 ± 4.8		20.96 ± 8.5	27.60 ± 15.1	0.001

A number of adipose tissue hormones have effects on the skeleton and vice versa. For example, leptin positively influences the differentiation of stem cells into osteoblasts, it inhibits RANKL, increases production of osteoprotegerin [11], while adiponectin has opposing effects: besides pro-osteoclast effect due to activating sympathetic nervous system, it also inhibits differentiation of osteoclasts. The level of adiponectin is negatively associated with BMD. Leptin and ghrelin level decreases after weight loss [12]. Adipose tissue also produces proinflammatory cytokines, which have negative influence on the skeleton through various mechanisms.

The study of endocrine interactions between adipose and bone tissue is a highly topical issue. This mutual communication represents a feedback homoeostatic system in which adipokines and molecules secreted by osteoblasts and osteoclasts are the connecting link of active axes fat – bone tissue [13]. However, for the most part, the mechanisms of this axis remain unknown [14]. Generally established knowledge that higher weight is one of the preventive factors against osteoporosis does not appear very conclusive. In recent years, studies have shown that surprisingly, osteopenia or osteoporosis can be found in some obese patients [15].

4 Conclusions

Effects of substantial weight loss on bone mass in morbidly obese patients have not been widely studied so far. The data that we present, has not yet been presented with respect to the obese yet, and it shows high dynamics of changes in bone metabolism during rapid extreme weight loss in highly obese individuals.

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P10 BEAD-BASED IMMUNOSENSOR FOR EARLY STAGE DIAGNOSIS OF OVARIAN CANCER

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Summary
New electrochemical immunomagnetic sensor for detection of human epididymal protein 4 (HE4), sensitive and selective biomarker of ovarian cancer, is presented. The sensor based on the combination of highly specific immunochemical reaction, easy handling magnetic separation and sensitive electrochemical detection allows evaluating of HE4 level at early stage of disease.

1 Introduction

Ovarian cancer is the fifth leading cause of all cancer related mortality among women. It is a treatable disease when diagnosed at early stage. Whereas the ovarian cancer is asymptomatic at early stages, most patients show advanced disease when diagnosed [1]. Therefore, the effort of many researchers is currently to use the combination of many biomarkers, which reflects the biological state [2].

Currently, the most used biomarker for ovarian cancer detection is serum cancer antigen 125 (CA125). Although, this biomarker with 72% sensitivity at specificity of 95% [1] is often used, human epididymal protein 4 (HE4) is recently of great importance as potentially new biomarker of ovarian cancer. HE4 is more specific than CA 125 in benign and malignant conditions and it´s level in human blood correlates with the stage of the disease [3]. Therefore, HE4 presents potentially powerful biomarker for detection of ovarian cancer at early stage, ideally before the occurrence of clinical symptoms.

Most of detection techniques of serum HE4 level are based on ELISA. In our work we combine the advantages of ELISA method, immunomagnetic separation and sensitive electrochemical detection using miniaturized screen-printed three-electrode sensors.

In this contribution magnetic particles with anti-HE4 antibodies ensure the capturing of the antigen from complex matrix. The formed immunocomplex is visualized by secondary antibodies labeled with alkaline phosphatase. This combination represents powerful device suitable for biomarker detection at very low concentration levels which is important for early stage disease diagnosis and treatment.

2 Experimental

2.1 Antibodies immobilization and immunomagnetic separation

100 µg of specific monoclonal anti-HE4 antibodies (Sino Biological, USA) were immobilized onto surface of 1 mg of magnetic particles Sera-Mag® with carboxylic functional groups (Thermo Scientific, USA) by standard carbodiimide method in 50mM MES pH 5.0 overnight upon gently mixing. The immobilization efficiency was examined by SDS-PAGE with silver staining method. Subsequent immunomagnetic separation of HE4 was performed in 0.1M phosphate buffer pH 7. The formed immunocomplex was visualized by secondary antibodies anti-HE4 labelled with alkaline phosphatase.

2.2 Voltammetric measurements

All voltammetric measurements were performed with interface PalmSens (PalmSens, Netherlands) with three-electrode sensors comprising carbon working and auxiliary electrode and Ag/AgCl reference electrode (DropSens, Spain) in order to miniaturize the sample volume up to 50 µl.

Hydroquinone diphosphate (HQDP) and p-aminophenyl diphosphate (PAPP), both in concentration 3mM in 0.1M Tris-HCl pH 9, were tested as the suitable substrates for enzymatic reaction with alkaline phosphatase (ALP). The resulting hydrolytic and electrochemically active product was detected by square-wave voltammetry (SWV).

3 Results and Discussion

The aim of the presented work was to develop highly selective and sensitive sensor for monitoring of serum levels of human epididymal protein 4 (HE4), new biomarker of ovarian cancer. This protein is going to be promising for early stage detection of ovarian cancer, because its level in blood correlates with the stage of the cancer.

The system employs advantages of immunomagnetic separation, which overcomes the drawbacks of direct immobilization of antibodies onto surface of miniaturized three-electrode sensors, such as low reproducibility and allows target preconcentration and purification.

Because specific anti-HE4 secondary antibodies labeled with ALP are not commercially available, these antibodies were prepared using commercial labelling kit with use of polyclonal anti-HE4 IgG antibodies (Innova Biosciences, UK). The labelling efficiency was verified by SDS-PAGE with silver staining method and the conjugate dilution was optimized electrochemically. All electrochemical measurements were performed using square-wave voltammetry as the sensitive electrochemical detection technique for detection of hydrolytic products of specific substrates of alkaline phosphatase (ALP). PAPP and HQDP were tested as potentially suitable substrates for above mentioned enzyme used as label of secondary antibodies. The highest response of electrochemically active products of hydrolytic reaction of alkaline phosphatase was gained using the substrate PAPP.

Although this system offers good sensitivity, the signal amplification allowing detection of lower concentration of HE4 is currently in progress.

4 Conclusions

Presented work is combination of highly specific ELISA method with magnetic separation and sensitive electrochemical detection. This system is suitable for reproducible detection of HE4 in complex matrix and with slight modification could be used for detection of other biomarkers at very low levels.

Acknowledgement

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P11 USE OF PCR-DGGE FOR CONTROL OF BACTERIA IN CHEESES AND THEIR PICKLES

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Summary

Denaturation gradient gel electrophoresis (PCR-DGGE) was used for control of bacteria in brained cheeses and their pickles. Composition of polymerase chain reaction (PCR) mixture and DNA amplification conditions were optimized for PCR with CG clamp primers. Phenol extraction and magnetic microspheres were used for purification of amplicons. Different numbers and intensities of amplicons reflect different bacterial composition in tested cheeses and pickles.

1 Introduction

Sheep, goat and cow milk brained cheeses are characterized by high content of salt. Salt is known by conservation effect, especially protection from mold [1]. The application of molecular biological methods (including comparison of 16S rDNA sequences) is useful for the understanding of microbial diversity. DGGE and 16S rDNA amplicons sequencing enable to detect culturable, uncultivable or unknown species of microorganisms in different complex samples [2].

2 Experimental

Analyzed samples were brained cheeses and their pickles. DNA was isolated by phenol chloroform extraction [3]. Concentration and purity of DNA was determined by UV spectrometry. The integrity of DNA was confirmed by agarose gel electrophoresis using 0.8% gel. PCR was performed with primers specific to domain *Bacteria* [4]. Specific products were detected by agarose gel electrophoresis with 1.8% gel. DNA marker 100 bp ladder for gel electrophoresis was from Malamité (Moravské Prusy, ČR). The PCR products were purified by magnetic microspheres poly(glycidyl methacrylate) – PGMA [5] and by phenol chloroform extraction [3]. DNA was eluted to 25 µl TE buffer overnight. PCR products with GC clamp were analyzed by DGGE INGENYphorU instrument (Goes, Neatherlands). PCR products (10 µl) were applied on 40-60% gradient gel and separated at 100 V for 10 minutes and at 60 V for 22 hours. The DNA was stained with ethidium bromide (0.5 µg/ml) and photographed at 305 nm. Visualised bands were cut from the gel and DNA eluted in 50 µl of TE buffer (10mM Tris–HCl, 1mM EDTA; pH 7.8) [6].

3 Results and Discussion

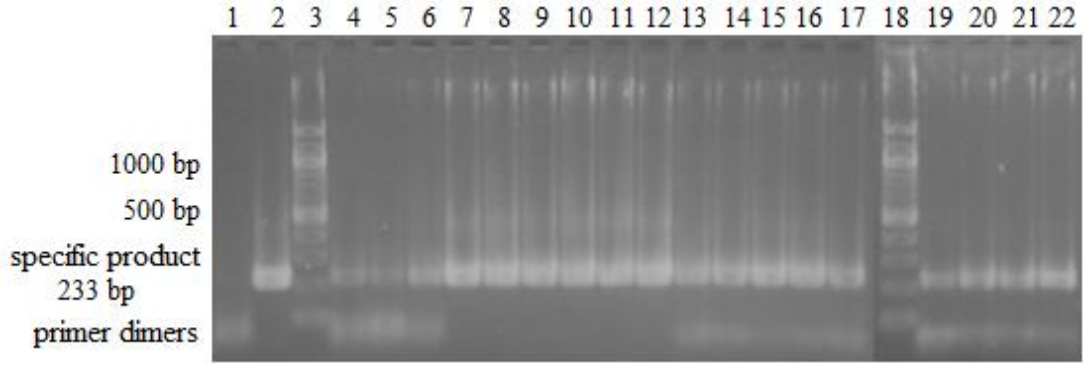
DNAs from real samples of cheeses and their pickles were isolated by phenol extraction in quality suitable for PCR. DNA was diluted to the concentration approximately 100 ng/µl.

In the next step, the amounts of Mg^{2+} ions, dNTP, primers and DNA polymerase were optimized in PCR mixtures. Sequences of primers F357 GC and R518 [4] is shown in Table 1.

Table 1. Sequences of primers with GC clamp.

Primer	Sequence 5'–3'	PCR product (bp)
F 357 GC	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG	233
R 518	ATTACCGCGGCTGCTGG	

Efficiencies of amplicon purification by phenol extraction and by magnetic microspheres were compared. Higher concentration of PCR products was obtained after reamlification of DNA purified by magnetic microspheres. Results of PCR products gel electrophoresis are given in Figure 1. PCR products (233 bp) were amplified in intensities suitable for next analysis. Three PCR products had lower intensity (runs 4-6).



Lane	DNA	Intensity of PCR products	Lane	DNA	Intensity of PCR products
1	Negative control	–	12	L 6	+++
2	Positive control	+++	13	KS 1	++
3	ladder		14	KS 2	+++
4	KL 1	+	15	KS 3	+++
5	KL 2	+	16	S 1	+++
6	KL 3	++	17	S 2	+++
7	L 1	+++	18	ladder	
8	L 2	+++	19	S 3	++
9	L 3	+++	20	S 4	++
10	L 4	+++	21	S 5	++
11	L 5	+++	22	S 6	+++

+, ++, +++ low, median and high intensity of PCR products, KL and L pickle, KS and S cheese

– PCR products were not detected

Fig. 1. Agarose gel electrophoresis of PCR products with primers with GC clamp (233 bp).

The PCR products with GC clamp (233 bp) were analyzed by DGGE. Results of the gradient gel electrophoresis are shown in Figure 2. There were fragments of approximately the same intensity in cheeses. The main difference in the number of fragments was among pickles. It reflects different bacterial compowsition of pickles with defect.

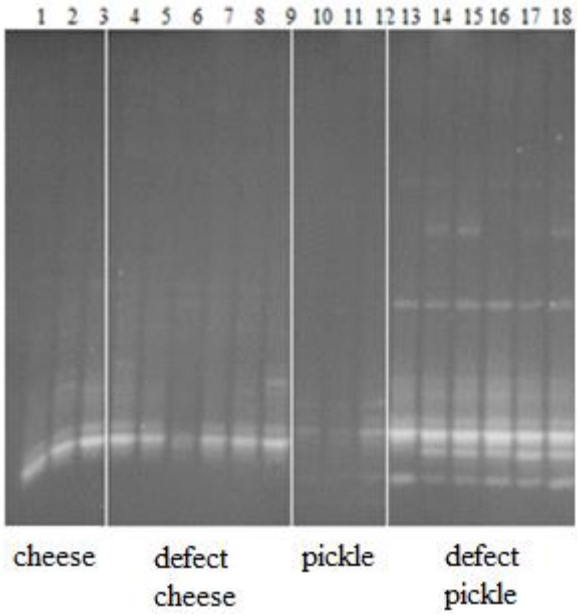


Fig. 2. Denaturation gradient gel electrophoresis of PCR products from cheese and their pickles. Lanes 1-9 cheeses, lanes 10-18 pickles.

4 Conclusion

It has been shown that the method of PCR-DGGE is suitable for the analysis bacteria in cheeses and their pickles.

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P12 PHOTO-INDUCED FLOW-INJECTION DETERMINATION OF NITRATE IN WATER

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Summary
A sensitive flow-injection method for the chemiluminescent determination of ultra-low concentration of nitrate in water is presented. Nitrate is on-line photolytically converted to peroxynitrite by absorption of UV light inside of 60 mm long quartz capillary (i.d. 530 μm, o.d. 720 μm). Peroxynitrite is subsequently determined by the chemiluminescent reaction with luminol.

The detection limit of nitrate is 7×10^{-10} M (S/N = 3). Linear range of the method is 2×10^{-9} to 1×10^{-5} M nitrate. The interference of nitrite is eliminated by its conversion to nitrogen after mixing of sample with a solution of sulfamidic acid. Other common anions do not interfere. The interference of cations is eliminated by passing the sample through a cation-exchange column. FIA procedure allows analysing of 15 samples per hour. The method was applied to the determination of nitrate in various real water samples. The results are in good agreement with a reference ion chromatographic method.

1 Introduction

Nitrates are widely spread contaminants of aqueous environments coming largely from agricultural application of fertilizers as well as from fabrication of drugs, explosives and other industrial products [1,2]. They are natural constituents of plants and important inorganic components of acid rains [3,4]. However, nitrates are not directly toxic to man but their possible reduction to nitrites and a next reaction of nitrites with secondary or tertiary amines present in the body can result in the formation of carcinogenic nitrosamines. Moreover, nitrites at high concentration can react with hemoglobin resulting in methemoglobinemia [5].

Present paper describes the ultra-sensitive determination of nitrate employing the photolysis of nitrate in a single quartz capillary using the high-pressure mercury lamp directly to peroxynitrite that is immediately detected by the chemiluminescent reaction with luminol. Compared to previous method [6,7], the newly proposed method allows faster, simpler and more sensitive detection of nitrate, together with effective elimination of known interferences from metal cations and nitrite.

2 Experimental

A schematic diagram of FIA system used for the determination of nitrate is shown in Fig. 1.

- The sample is sucked first through the cation-exchange column (Dowex® 50W-X_2) and then through the sample loop of an automatically operated six-way injection valve that introduces the sample into the carrier stream (destilled/deionized water).
- The carrier stream is merged with a reagent solution (8 mM sulfamidic acid (SMA), 1% v/v acetone and 0.6% v/v formaldehyde).
- During the flow through a reaction coil (PTFE, 0.75 mm i.d. × 9 m length), nitrites that interfere at nitrate detection are removed by their reaction with SMA according to reaction: $NO_2^- + NH_2SO_3H \rightarrow N_2 + HSO_4^- + H_2O$
- The aqueous mixture is then transported through the photolytic converter (high-pressure Hg lamp - 125 W, 192±0.5 °C) where nitrate is converted into peroxynitrite that is merged directly inside a chemiluminescent detector with a CL solution (1 mM luminol and 10 mM sodium pyrophosphate in 0.1 M KOH). The addition of small amount of acetone into nitrate solution increased the stability of peroxynitrite resulting in increased CL signal nearly by 3 orders.
- Emitted CL light detected by photomultiplier tube corresponds to nitrate concentration.

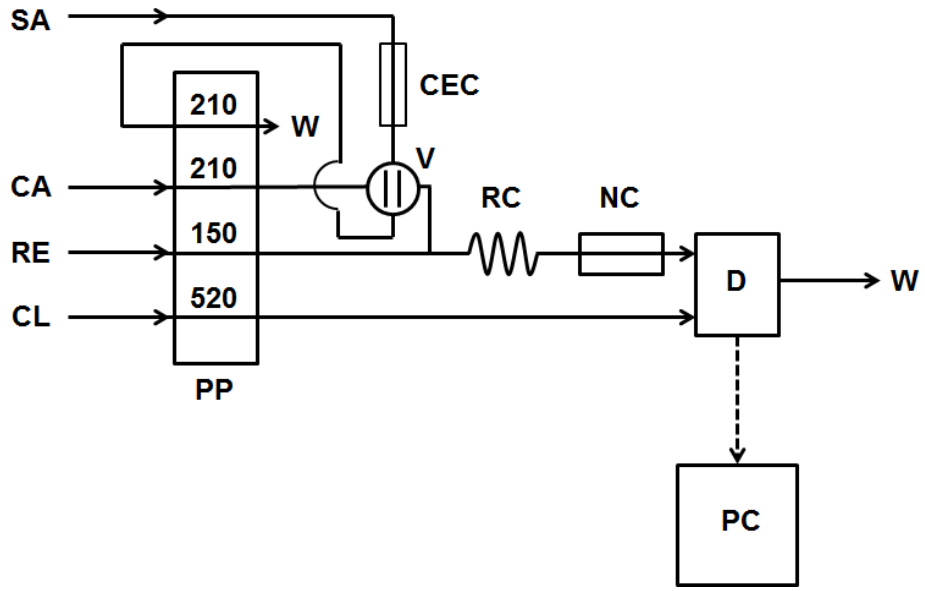

Fig. 1. Scheme of FIA apparatus. SA – sample (F = 210 µL min^{-1}), CA – carrier (F = 210 µL min^{-1}), RE – reagent solution (F = 150 µL min^{-1}), CL – chemiluminescent solution (F = 520 µL min^{-1}), PP – peristaltic pump, W – waste, CEC – cation-exchange column, V – injection valve, RC – reaction coil (PTFE tube with ID 0.75 mm and length 9 m), NC – nitrate photolytic converter, D - chemiluminescent detector, PC – computer.

3 Results and Conclusions

It was developed sensitive flow-injection method for the chemiluminescent determination of ultra-low concentration of nitrate in water.

- The detection limit of nitrate is 7×10^{-10} M (S/N = 3). Linear range of the method is 2×10^{-9} to 1×10^{-5} M nitrate.
- The relative standard deviation for 10 measurements is 2.1% at 1×10^{-6} M and 4.3% at 1×10^{-7} M NO_3^-, respectively.
- The interference of nitrite is eliminated by its conversion to nitrogen after mixing of sample with a solution of SMA. Other common anions do not interfere. The interference of cations is eliminated by passing the sample through a cation-exchange column.
- Analysis time of method is 11 min, FIA procedure allows analysing of 15 samples per hour.
- The method was applied to the determination of nitrate in various real water samples (Tab. 1).
- The results are in good agreement with a reference ion chromatographic method.
- Absence of toxic copperized cadmium (used for nitrate conversion) makes the developed method friendly to environment and offers it's using as a good alternative to current FIA methods for the determination of nitrates in different kinds of water samples as well as at various monitoring systems.

Table 1. Determination of nitrates in real water samples.

Sample	$[NO_3^-]$ (mg L^{-1})		%
	FIA	IC	difference
Drink water (no. 1)	33.5 ± 1.5	29.0 ± 0.3	+ 15.5
Drink water (no. 2)	31.6 ± 0.3	28.2 ± 0.3	+ 12.1
River water (no. 1)	7.39 ± 0.2	7.96 ± 0.3	– 7.16
River water (no. 2)	2.31 ± 0.1	2.29 ± 0.2	+ 0.87
River water (no. 3)	7.06 ± 0.3	7.20 ± 0.3	– 1.94
Well water	34.0 ± 1.7	33.1 ± 0.4	+ 2.72

Acknowledgement

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P13 OPTIMIZATION OF AMINO ACID DERIVATIZATION PROCEDURE USING NAPHTHALENE-2,3-DICARBOXALDEHYDE FOR CAPILLARY ELECTROPHORESIS WITH FLUORESCENCE DETECTION

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Summary
Amino acids (AAs) are very important compounds in living organisms and their quantification in biological fluids can be used for diagnostic purposes. For determination of AAs by capillary electrophoresis coupled with laser induced fluorescence detection (CE-LIF), derivatization procedure is necessary because only a few AAs exhibit native fluorescence. Derivatization reagent naphthalene-2,3--dicarboxaldehyde (NDA) reacts with primary amines in the presence of nucleophile, such as cyanide anion, to form fluorescent derivates. Derivatization reaction can be done in pre-capillary or on-capillary arrangement. In on-capillary arrangement the capillary is used both for separation and derivatization. Optimization of pre-capillary and on-capillary derivatization procedure of AAs using NDA and NaCN, and comparison of these approaches were performed.

1 Introduction

AAs play a significant role in living organisms. Quantification of AAs in biological fluids can reveal health-state of an organism thus can be used for diagnostic purposes. CE-LIF is a powerful and sensitive analytical technique suitable for determination of AAs. Only a few AAs exhibit native fluorescence and thus derivatization procedure is necessary. NDA reacts in the presence of nucleophile, such as cyanide anion, with primary amines to form fluorescent derivates [1]. Derivatization reaction can be performed pre-capillary or on-capillary. These approaches were optimized and compared.

2 Experimental

2.1 Model sample

Model sample was composed of 19 standard AAs and internal standard (IS) norleucine (nLeu) dissolved in water. Concentrations of AAs and IS were 10 μM and aliquots were stored frozen at -20 °C. The aliquot was thawed at a room temperature on the day of use.

2.2 Method

The Agilent G7100A CE System (Agilent Technologies, Santa Clara, CA, USA) coupled with external ZETALIF™ LED 480 detector (Picometrics, Toulouse, France) using λ_{ex} 480 nm was utilized in this study. The photomultiplier tube (PMT) voltage was 650 V and rise time (RT) was set to 0.1 second. Separation conditions were based on the article published by *Siri et al.* [2] and modified to use sodium instead of lithium salts in background electrolyte (BGE). BGE was composed of 30 mM sodium tetraborate and 40 mM sodium dodecyl sulfate (SDS). 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 71.0 cm total length (L), 50.0 cm effective length (L_{ef}) and 50/375 μm inner/outer (ID/OD) diameters was used. Capillary was kept at 25 °C and separation voltage was 30 kV. Sample was introduced into capillary using pressure of 25 mbar for 4 seconds in the case of pre-capillary derivatization. Sequential injection of 11 mM NaCN, sample of AAs and 1 mM NDA by pressure of 25 mbar for 6 seconds each zone was used in the case of on-capillary derivatization.

2.3 Benzoine condensation

Siri et al. [2] proposed the derivatization procedure using derivatization mixture prepared in advance and consisted of NDA, KCN and sodium tetraborate buffer. It is known, that NDA reacts in the presence of cyanide to form fluorescent NDA condensates [3]. These reasons lead to necessity of optimization this derivatization procedure with an emphasis on the preparation of derivatization mixture freshly just before the derivatization.

3 Results and Discussion

3.1 Pre-capillary derivatization

The composition of derivatization mixture consisted of NDA, NaCN and sodium tetraborate buffer was optimized. It was found, that concentration of sodium tetraborate does not affect the reaction outcomes significantly, but sufficient buffer capacity is necessary for derivatization of biological samples, e.g. for urine samples. Concentrations of NDA and NaCN affect both derivatization outcome of AAs and the production of fluorescent NDA condensates (data are not shown). Final concentrations of NDA and NaCN as well as reaction time and temperature were chosen in respect of yield of the derivatization reaction and amount of formed fluorescent NDA condensates. Derivatization mixture was composed of 0.1 mM AA (sum of AAs), 0.5 mM NDA, 1 mM NaCN and 50 mM sodium tetrabotrate. Derivatization reaction was done at thermomixer at 25 °C and 650 rpm. Reaction time

was 20 minutes. Stability of NDA derivates of AAs was studied at laboratory temperature. Sample was repeatedly analyzed in 30 minutes intervals and the stability was evaluated as the change in the peak area over time. Degradation profiles were obtained and the speed of degradation ranged from 1.7-5.7 % per hour. Instability of NDA derivates lead to impossibility of automated analyzes of biological samples.

3.2 On-capillary derivatization

Similarly to the pre-capillary derivatization, the concentrations of NDA, NaCN, sodium tetraborate buffer and reaction time were optimized. Ratios of zone lengths of reagents introduced into the capillary by sequential injection were optimized as well. Concentration of 100 mM sodium tetraborate was chosen because of preservation sufficient buffer capacity. Results of optimization of NDA and NaCN concentrations are shown in the Fig. 1.

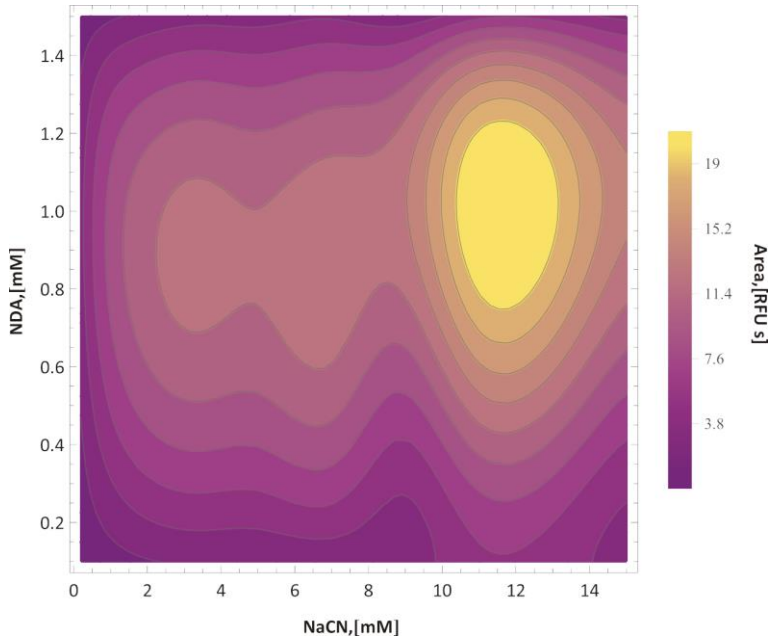

Fig. 1. The contour graph showing results of optimization of NDA and NaCN concentrations. Concentration of sodium tetraborate was 100 mM. Sequential injection: NaCN, model sample, NDA by pressure of 10 mbar for 5 seconds each zone; reaction time: 5 minutes; BGE: 30 mM sodium tetraborate, 40 mM SDS; capillary: 50/375 µm ID/OD, 71/50 cm L/L_{ef}; voltage: 30 kV; temperature: 25 °C; detector: ZETALIF™ LED 480, λ_{ex} 480 nm, PMT 650 V, RT 0.1 second.The color range illustrates the average reaction outcome. The yellow area represents the highest yield and was considered as reaction optimum.

Final derivatization conditions were chosen in respect of yield of the derivatization reaction and total analysis time. Amount of formed NDA condensate was significantly reduced because derivatization procedure is immediately followed by separation of reaction mixture. Derivatization mixture was composed of 0.2 mM AA (sum of AAs), 1 mM NDA, 11 mM NaCN and 100 mM sodium tetraborate. The reaction was done on-capillary at 25 °C. Reaction time was 5 minutes.

3.3 Comparison

The quantitative comparison of pre-capillary and on-capillary derivatization procedures was not possible because of different reaction time and dilution. The comparison was based on repeatability, day-to-day repeatability and technical performing. Repeatability and day-to-day repeatability were measured and results are listed in Tab. 1. On-capillary derivatization proved to be better in the case of repeatability and day-to-day repeatability in the peak areas about 2× and 2.4×, respectively.

Table 1. Repeatability and day-to-day repeatability of chosen NDA derivates of AAs.

Validation	**Repeatability (n=6)**				**Day-to-day repeatability (n=6)**			
Mode	Pre-capillary		On-capillary		Pre-capillary		On-capillary	
Derivates of AAs	RSD of time, %	RSD of area, %	RSD of time, %	RSD of area, %	RSD of time, %	RSD of area, %	RSD of time, %	RSD of area, %
His	0.42	4.50	0.38	1.82	0.37	8.33	0.62	3.48
Ala	0.41	5.82	0.37	3.25	0.37	8.96	0.94	3.73

4 Conclusions

Optimizations of pre-capillary and on-capillary derivatization procedures of AAs were performed. On-capillary derivatization proved to be better in the case of repeatability and day-to-day repeatability in the peak areas. It is methodically easier to perform because of possibility of automation and it is considered to be more precise due to reduction of manual steps. Other advantage of on-capillary is a necessity of smaller volumes of sample and other reagents compared with pre-capillary arrangement. Pre-capillary derivatization is quite time consuming, and the instability of NDA derivates most likely cause higher than 4% and 8% RSDs of peak areas for repeatability and day-to-day repeatability, respectively. Routine automated analyses are thusly hindered.

Acknowledgement

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P14 MODELLING OF MICROFLUIDIC FLOW-GATING INTERFACE FOR TWO-DIMENSIONAL LIQUID CHROMATOGRAPHY-CAPILLARY ELECTROPHORESIS

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Summary
In this work, the finite element approach was used for the numerical evaluation of the fraction transfer process in on-line liquid chromatography-capillary electrophoresis two-dimensional system. The results of the modelling of microfluidic flow-gating interface were compared with the experimental data obtained during transfer of dye zone through the interface.

1 Introduction

The on-line coupling of liquid chromatography (LC) with capillary electrophoretic (CE) separations is not a trivial task as it requires compatibility of mobile phases with background electrolytes, matching capillary and column dimensions and last but not least proper electrical connection. The most commonly used design of the interface for two-dimensional LC-CE is based on the flow-gating process [1,2]. The valve-based interfaces are less frequently utilized [3]. Besides the aforementioned solutions, which can employ commercially available instrumentation, the microchip format of the part [4], or the whole two-dimensional separation system has been recently reported [5]. In this work, the flow-gating-type of the interface was modelled and the fraction transfer process was simulated using finite element approach taking into account hydrodynamic flow and diffusion of zones of the analytes. The results were compared with the real experiment of dye transfer in microfluidic interface fabricated in glass.

2 Experimental

2.1 Flow-gating interface

The interface was fabricated in borosilicate glass using photolithography and wet etching procedure as described elsewhere [6]. The interface design (14 x 14 mm) consisted of two channels perpendicular to each other (approx. 380 μm i.d.). The interface was placed in the 3D printed support frame constructed from polylactic acid. The 50 μm i.d. capillaries (Agilent, Palo Alto, CA, USA) with the grinded tips to the 45° angle with respect to the longitudinal axis representing capillary with LC effluent and CE separation capillary were mounted in the longitudinal position to the interface

with defined gap between their tips and sealed there using a silicone septum. The other two channels were connected to the 250 mL gastight syringe (Hamilton, Bonaduz, Switzerland) filled with background electrolyte and to the short 100 μm i.d. fused silica capillary used as the waste, respectively. The NE-100 syringe pump (New Era Pump Systems, Farmingdale, NY, USA) provided the transverse gating flow of background electrolyte.

2.2 Finite element modelling

For the modelling of the processes connected with the fraction transfer, the mesh of the elements was created based on the physical dimensions of the interface using Gmsh 2.8.4 software employing Delaunay meshing algorithm [7]. The finite element method modelling of the interface was carried out in the Elmer 7.0-rev6064 software (www.csc.fi/elmer). The boundary conditions for the calculations were defined in the term of velocity flow profile and the non-slip wall conditions were assumed. The results were visualized using Elmer VTK build-in postprocessor and ParaView 4.2.0-RC1 software (www.paraview.org).

3 Results and Discussion

On-line coupling of capillary liquid chromatography with capillary electrophoresis using flow-gating interface was modelled in three steps. The calculated results were verified by measuring of the dispersion of the methylene blue dye solution during the fraction transfer process. At the first, the LC effluent flow at the inlet capillary of the interface was characterized. The velocity of the flow rapidly decreases with increasing distance from the capillary outlet yielding significantly broadened zone of the analytes with the longer gap than 50 μm, which corresponds with the internal diameter of the capillary (Figure 1).

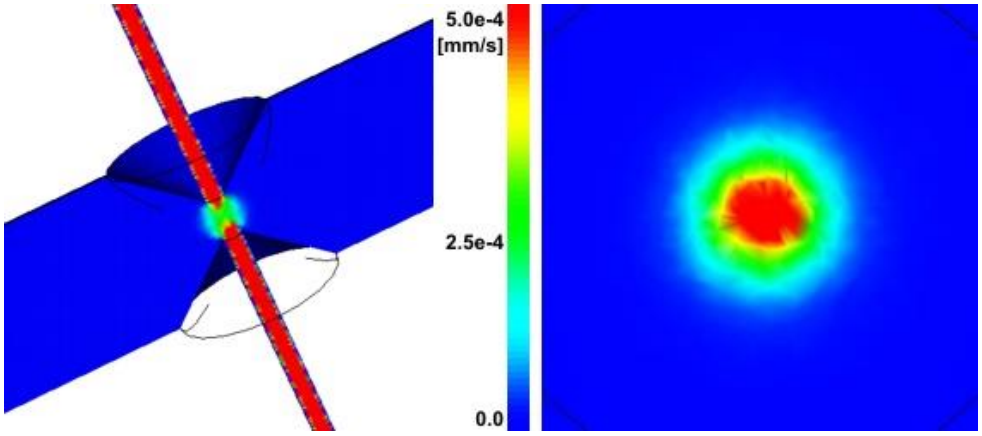

Fig. 1. Velocity flow profile during the fraction transfer process (left) and radial distribution of the flow at the 50 μm distance from the capillary outlet (right).

The second step consisted of the calculation of conditions for the transverse flow of background electrolyte. Using the effluent capillary with grinded edges to prevent the bubble formation, the velocity flow profile inside of the gap is reduced

and should be compensated with the increasing overall transverse flow of the electrolyte.

In the last part of the work, the injected profile at the inlet of electrophoretic capillary together with the gating process was modelled. The length of the injected zone is determined by the width of the analyte zones in LC effluent together with the time of the electrophoretic separation and yielded approx. sampling rate of one fraction per peak under tested conditions (average width of the peak in half height $w_{1/2}$ = 20 s; -30 kV separation voltage; lowest effective mobility of the analyte $-20 \cdot 10^{-9}$ $m^2V^{-1}s^{-1}$; eliminated electroosmotic flow). The gating flow rate velocity profile in longitudinal and radial direction with respect to the separation capillary is sufficient for closing of the interface is shown in Figure 2.

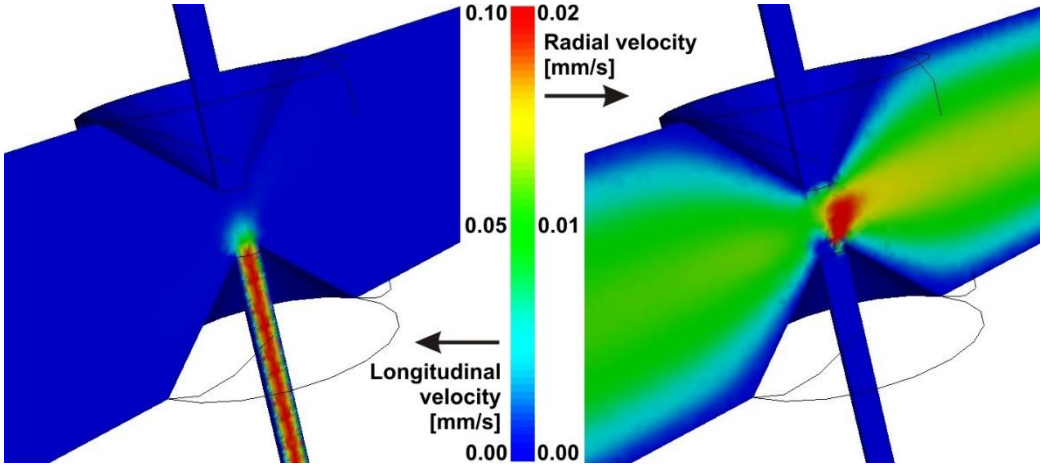

Fig. 2. Longitudinal and radial velocity flow profile inside of the flow-gating interface.

4 Conclusions

The processes connected with the on-line coupling of liquid chromatography with the capillary electrophoresis were modelled using finite element method. The optimal working conditions were estimated taking into account hydrodynamic flow of both LC effluent and transverse gating flow of background electrolyte. The modelled fraction transfer process allowed the compensation for the changing viscosity and density of the LC effluent introduced by gradient elution.

Acknowledgement

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P15 INFLUENCE OF PEG 6000 CONCENTRATION ON DNA VISCOSITY AND ADSORPTION CAPACITY ON MAGNETIC MICROSPHERES

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Summary
The aim of this work was to evaluate the influence of (PEG 6000) concentration on DNA viscosity and adsorption capacity of hydrophilic magnetic non-porous microspheres P(HEMA-*co*-GMA) coated with carboxyl groups. Magnetic particles were separated from the mixture and DNA was eluted from particles. More pronounced DNA adsorption was achieved at critical and higher PEG 6000 concentrations.

1 Introduction

Isolation of nucleic acids is the basic step in molecular biotechnology. High DNA quality is required for the majority of applications. There is a lot of methods for DNA isolation. Magnetic particles have been attractive for DNA isolation from the various types of samples [1]. Manipulation with magnetic particles is very easy. They can be quick and simple removed from the reaction mixture. The aim of the first part of work was focused on the study of DNA viscosity in the mixture. The second part was focused on the application of solid magnetic microspheres for DNA isolation.

2 Experimental

Calf thymus DNA was purchased from Sigma Aldrich. Other chemicals were in p.a. quality. Hydrophilic magnetic non-porous microspheres poly(2-hydroxyethyl methacrylate-*co*-glycidyl methacrylate) P(HEMA-*co*-GMA) were prepared as described earlier [2]. Composition of mixtures for viscosity determination is given in the Table 1. The volume of magnetic microsphere was replaced with sterile water.

Temperature 25°C was used for determination of viscosity versus PEG 6000 concentration. The viscometer SV-10 Vibro Viscometer (A & D Company Ltd., Tokyo, Japan) was used for viscosity measurements.

Demineralized water, sodium chloride (2 M), poly(ethylene glycol) 6000 (PEG 6000) (40 %), calf thymus DNA (2,000 ng·µL^{-1}) in TE buffer (10 mM Tris-HCl, pH 7,8; 1 mM EDTA, pH 8,0) and magnetic microspheres (2.0 mg·mL^{-1}) were mixed together according to Table. 1. Final concentration of DNA in the mixture was 200 ng·µL^{-1}. After 60 minutes, magnetic microspheres with DNA were separated using magnetic separator. Supernatants were removed, particles with DNA were washed two times with 70 % ethanol and dried in exicator at laboratory temperature. TE buffer (50 µL) was used for DNA elution (60 minutes). UV/Vis NanoPhotometer (Implen, München, Germany) was used for UV spectrophotometry.

Table 1. Mixture for DNA separation.

Components (µL)	% PEG 6000 (w/v)					
	2	4	6	8	10	16
H_2O	325	300	275	250	225	150
NaCl (5 M)	50	50	50	50	50	50
DNA (2 000 ng/µL)	50	50	50	50	50	50
PEG 6000 (40%)	25	50	75	100	125	200
Magnetic microspheres (2 mg·mL^{-1})	50	50	50	50	50	50

3 Results and Discussion

Exponentially viscosity increase was detected with linear increase of PEG 6000 concentration. Results are given in Fig. 1.

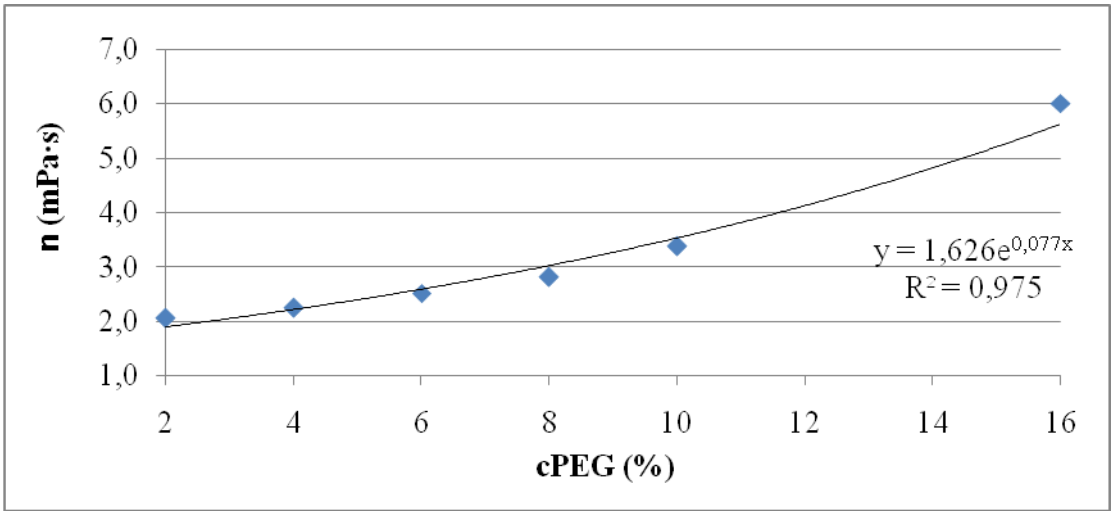

Fig. 1. Influence of PEG 6000 concentration on viscosity of DNA separation (25°C).

The same mixtures (Table 1) were used for DNA separation. Concentration of PEG 6000 affected the sorption of DNA on particles surface (Fig. 2). Linear increase of PEG 6000 concentration caused dramatic change in concentration of eluted DNA. The expressive difference was detected between 6 and 8 % of PEG 6000. Condensation of DNA occurred under these experimental conditions [3]. Condensed DNA was adsorbed on the surface of solid magnetic microspheres.

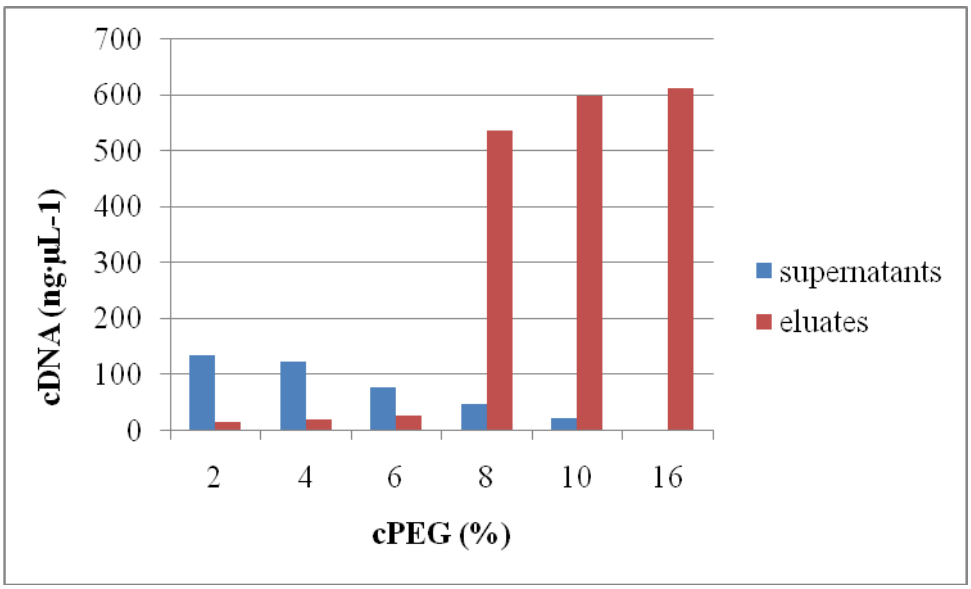

Fig. 2 Influence of PEG 6000 concentration on DNA concentration in supernatants and eluates.

4 Conclusions

The results presented in this report show that hydrophilic magnetic non-porous P(HEMA-*co*-GMA) microspheres containing carboxyl groups are suitable for isolation of calf thymus DNA. More pronounced DNA adsorption was achieved at critical and higher PEG 6000 concentrations. Condensed DNA was obviously adsorbed to the surface of microspheres.

Acknowledgement

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P16 MONOSACCHARIDE ANHYDRIDES IN ATMOSPHERIC AEROSOLS – OPTIMIZATION OF SEPARATION AND DETECTION BY MEANS OF LC-MS TECHNIQUE

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Summary
We report a simple analytical method, based on high-performance liquid chromatography with mass spectrometric detection (HPLC-MS), which was developed and utilized to determine monosaccharide anhydrides (levoglucosan, mannosan, and galactosan) in atmospheric aerosols. Prior to chromatographic separation optimized on columns with different chemistries, the effectiveness of the selected solid-phase extraction (SPE) materials to pre-concentrate of target compounds were compared.

1 Introduction

Chemical composition of atmospheric aerosols including high complexity of components varies significantly with time and place, which needs appropriate time-resolved detection. So far, low knowledge about organic fraction of aerosols has been obtained, especially water-soluble organic constituents are poorly characterized. Levoglucosan, mannosan, and galactosan are important representatives of water-soluble organic compounds in fine atmospheric aerosols. These monosaccharide anhydrides (MAs) are specific tracers and molecular markers of biomass burning, products of cellulose combustion or hemicellulose pyrolysis, emitted into the smoke aerosol. Levoglucosan is the most abundant individual organic compound identified in aerosols [1, 2, 3].

2 Experimental

The SPE methods for the selective concentration of MAs were examined on the set of commercially available and in laboratory prepared cartridges filled with different sorbents. Due to the polar character of analyts, hilic (diol, amino, cyano, polyamide, silica, chitosane), polar modified reverse and carbon based phase were evaluated and compared in terms of retention and recovery.

HPLC experiments were focused on the separation and resolution of levoglucosan, mannosan, and galactosan. To reach the aims, the chromatographic stationary phases tested were amido (TSKgel Amide-80; Tosoh Bioscience), amino (Prevail Carbohydrate ES; Grace), C18 (Poroshell 120SB-Aq; Agilent), porous graphite (Hypercarb; Thermo Scientific), and silica (Kinetex hilic; Phenomenex) in narrow bore columns. HPLC analysis were conducted on 1100 Series (Agilent) chromatograph hyphenated with electrospray ion-trap spectrometer Esquire LC (Bruker Daltonics).

MS conditions were optimized with the solutions of standard MAs to get the lowest limit of detections (LOD). The method development consists in post-column addition of appropriate solution enhancing ionization of target species, especially water with or without addition of CH_3COOH, CH_3COONH_4, NH_4Cl, LiCl, and NH_3 in various concentrations.

3 Results and Discussion

The comparison of the SPE column indicated that the majority of ones failed in the retention of MAs. The polar modified reverse phases (ENVI-chrom P, Isolute ENV+) show recovery about 3 %, while the application of carbon black with large surface area (Silcarbon K300) provides recovery up to 47% in the case of levoglucosan.

Chromatographic behavior resembles the observations from SPE experiments. Used reverse phases showed low retention and either no resolution of MAs (C18) or partial isolation of levoglucosan peak from others (graphite). To achieve sufficient resolution, hilic columns were used at high percentage of acetonitrile (95 % or more) as a mobile phase. However, baseline separation of isomers was obtained only for amino column.

No chromophore in carbohydrate molecule and the need of sensitive method lead to the preferential application of MS detector rather then refractometric or diode-array ones. Low abundance of ions corresponding to the target species due to the high organic mobile phase were eliminated by post-column addition of several water solutions (Fig. 1.). The highest increase in response ($[M+Li]^+$ ions) induced the addition of 1mM LiCl (up to 18 times in comparison with water addition). Under these conditions, LOD for mannosan and levoglucosan were approximately 3 ng. Comparable intensity of deprotonated molecules of all anhydrides showed the addition of 25mM ammonium hydroxide, however, with significant loss of intensity.

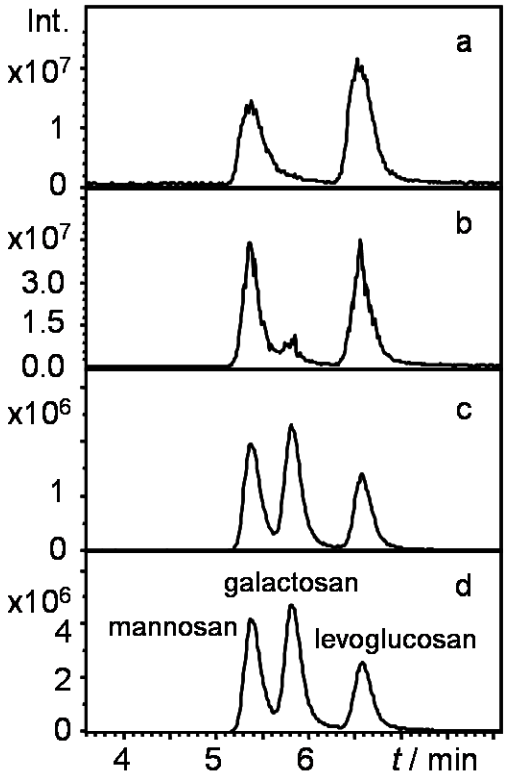

Fig. 1. Extracted ion chromatograms of ions corresponding to MAs after post-column addition of water (a, c), 1mM LiCl (b), and 25mM NH_3 (d) in the positive (a, b) and negative (c, d) ion mode.

4 Conclusions

Pre-concentration of monosaccharide anhydrides from atmospheric samples were performed using solid-phase extraction technique on active carbon. LC-MS method based on the separation on amino column was successfully developed and applied to the analysis of levoglucosan and other anhydrides. Detection issue has been overcome by the post-column addition of ammonium hydroxide or lithium chloride solutions.

Acknowledgement

This work was supported by Grant Agency of the Czech Republic (P503/14/25558S) and by the Institute of Analytical Chemistry of the ASCR under an institutional support RVO: 68081715.

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P17 EXTRACTION OF BIOAVAILABLE PROPORTION OF METALS IN DIESEL EMISSIONS AND ATMOSPHERIC AEROSOLS USING ARTIFICIAL LUNG FLUIDS

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Summary
The determination of bioavailable proportion of selected metals in biofuel exhaust and atmospheric aerosols, and a comparison of artificial lung fluids in relation to the yield of extraction were performed.

1 Introduction

The emissions from engines incorporate large amounts of metals which are contained directly in fuels or are used as engine oil additives. A degree of toxicity of metals in particulate matter (PM) cannot be evaluated on the basis of total concentrations; the determination of bioavailable proportion is necessary. To assess the bioavailability of metals, the extraction in solutions of simulated lung fluids may be used. However, artificial fluids used for several decades differ considerably in chemical composition.

2 Experimental

PM1 aerosol (D_p < 1 μm) was collected on nitrocellulose filters. Two filters with PM1 from the combustion of biofuel (WHTC 1 and WHTC 2) and two filters with PM1 from urban air (Brno 1 and Brno 2) were collected. Parts of samples were extracted in the following reagents: Gamble's solution – A, Gamble's solution with dipalmitoyl phosphatidylcholine (DPPC) – B, artificial lysosomal fluid – C [1], saline – E, deionized water – F, and newly proposed solution with DPPC simulating the extraction in alveoli (SEA) – D. Contents were determined by means of ICP-MS.

The main substance reducing the surface tension in alveoli is DPPC [2,3]. Therefore, SEA containing 0.1 g/L DPPC and 1.2 mmol/L Ca^{2+} (physiological concentration) [3] in a dilute phosphate buffer at pH = 7.4 was proposed.

3 Results and Discussion

Total contents of Al, Ce, Cr, Cu, Mn, Ni, Pb, V, Zn as well as bioavailable proportions were determined. The determination of bioavailable copper is presented in Fig. 1. The highest bioavailability was observed for vanadium, the bioavailability of other elements was substantially dependent on the composition of lung fluid simulant. The highest extraction efficiency evinced artificial lysosomal fluid (C)

while Gamble's solution (A) commonly used was considerably weaker extractant. Newly designed simulant of extraction in alveoli (D) had surprisingly lower extraction power than deionized water regardless of lower surface tension.

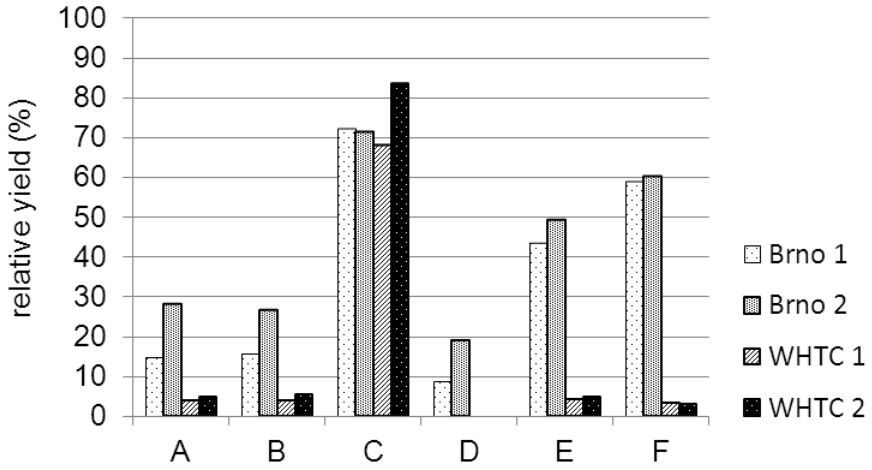

Fig. 1. Relative yields of copper extracted in simulated lung fluids.

4 Conclusions

Results evince, that simulated lung fluids differ significantly in extraction power, and therefore, a broad scientific discussion is necessary for further application in environmental science.

Acknowledgement

This work was supported by Grant Agency of the Czech Republic under grant No. P503/13/1438S and by Institute of Analytical Chemistry of the Academy of Sciences of the Czech Republic under an institutional support RVO: 68081715.

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P18 TOWARDS THE PRODUCTION OF HIGHLY EFFICIENT POLYMERIC MONOLITHIC COLUMNS FOR HILIC SEPARATIONS OF SMALL MOLECULES

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Summary
Short polymerization times, as well as secondary polymerization on scaffolds (such as silica particles) have shown great potential in the generation of highly efficient stationary phases. Short polymerization times have been shown to produce columns with high separation efficiencies of small molecules, relative to columns prepared from full conversion. Short polymerization times in the preparation of polymeric monolithic columns have predominantly focused on preparation from styrenic monomers. For the most part, columns prepared for HILIC applications consist of zwitterionic monomer and a cross-linker within a suitable porogenic system, and are prepared in a single step polymerisation. In this work, lauryl methacrylate-*co*-tetraethyleneglycol dimethacrylate monolithic scaffolds were prepared, using thermal polymerization at 60 °C with varying polymerization times. A subsequent secondary polymerization was performed using the zwitterionic monomer, N,N-dimethyl-N-metacryloxyethyl-N-(3-sulfopropyl) ammonium betaine, and the cross-linker bisphenol A glycerolate dimethacrylate, with thermal polymerization at 60 °C, and varying polymerization times. A number of columns were prepared, within the confines of a 0.320 mm i.d. fused silica capillary. A study into the effects of the short polymerization times was also completed. Characterisation was performed using permeability, porosity, inverse size exclusion, Van Deemter curves, and scanning electron microscopy. The formation of the scaffold monolith was optimized, prior to the investigation of the secondary polymerization. For the first time, a study on the effect of secondary polymerization of methacrylate monomers upon methacrylic monolithic columns has been performed. The resulting columns were used in the HILIC separation of model compounds, and phenolic acids, with at least 61, 000 N/m obtained in HILIC mode for thiourea. The effect of volume fraction of water within the mobile phase was investigated, exhibiting the expected dual retention mechanism characteristic of HILIC stationary phases. The generated efficiencies demonstrate the potential of short polymerization times in the preparation of polymeric monolithic columns.

Acknowledgements
This work was financially supported by the project Enhancement of R&D Pools of Excellence at the University of Pardubice, reg. Nr. CZ.1.07/2.3.00/30.0021.

P19 TESTING DIFFERENT TYPES LYSIS METHOD FOR THE PREPARATION OF THE BACTERIAL CELL LYSATES WITH SUBSEQUENT ISOLATION AND AMPLIFICATION OF DNA

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Summary
The aim of this work was the optimization of procedure the crude cell lysates preparation of *Lactobacillus* genus. In this experiment different lysis methods were tested: buffer B, 4% solution of washing powder Amway and using of immobilized lysozyme. At the same time a lysing reagent was tested. As a control sample was used a buffer A which contained no lysozyme. Crude lysates were used for a DNA isolation using magnetic microspheres. The DNA was examined by the PCR method.

1 Introduction

Preparation of high-quality DNA is an essential step in the molecular diagnostics of microorganisms. Procedure of crude cell lysates preparation play an important role in molecular diagnostics. In biological research enzymes and detergents have tradionally been used to disrupt cells. DNA is released into medium, it is available for purification and amplification by polymerase chain reaction (PCR).

2 Experimental

The *Lactobacillus paracasei* subsp. *paracasei* CCDM 212/106 strain was obtained from the Czech Collection of Microorganisms (CCM, Brno, Czech Republic).

At start, 1 ml of the overnight grown cell cultures (A_{600} nm = 3.00) was resuspended in 1000 µl lysis buffer A (10 mM Tris-HCl, pH 7.8; 5 mM EDTA, pH 8.0). The cell lysates preparation conditions were: a) 500 µl buffer B (10 mM Tris-HCl, pH 7,8; 5 mM EDTA; 3mg/ml lysosyme), b) 500 µl 4% solution of washing powder Amway, c) 490 µl buffer A a 10 µl immobilized lysozyme. As a control sample was used a buffer A which contained no lysozyme. The mixture was incubated for 1, 3 a 5 hours (room temperature). Then 25 µl 10 % SDS a 5 µl proteinse K (100 mg/ml) were added in some case and the mixture was incubated 55°C overnight.

The crude lysates were used for the DNA isolation using magnetic poly(2-hydroxyethyl methacrylate-co-glycidyl methacrylate) (P(HEMA-*co*-GMA) (1:1) microspheres covered by carboxyl groups (2,67 mM), particle diameter 2,2 µm, iron content - 6,5 %. The magnetic microspheres covered by lysosyme - poly-(2-hydroxyethyl methacrylate-*co*-glycidyl methacrylate) (P(HEMA-*co*-GMA)) (1:1) were also used. The microspheres were prepared by D. Horák from Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic. DNAs were isolated from crude cell lysates in solutions of 16 % PEG 6,000 and 2.0 M NaCl. The

presence of the target DNA was verified by PCR using LBLMA 1 and R16 primers specific to the *Lactobacillus* genus [1].

3 Results and Discussion

The PCR products were detected using agarose gel electrophoresis (1.8 %), 80 V, 2 hour). Results are given in Fig. 1.

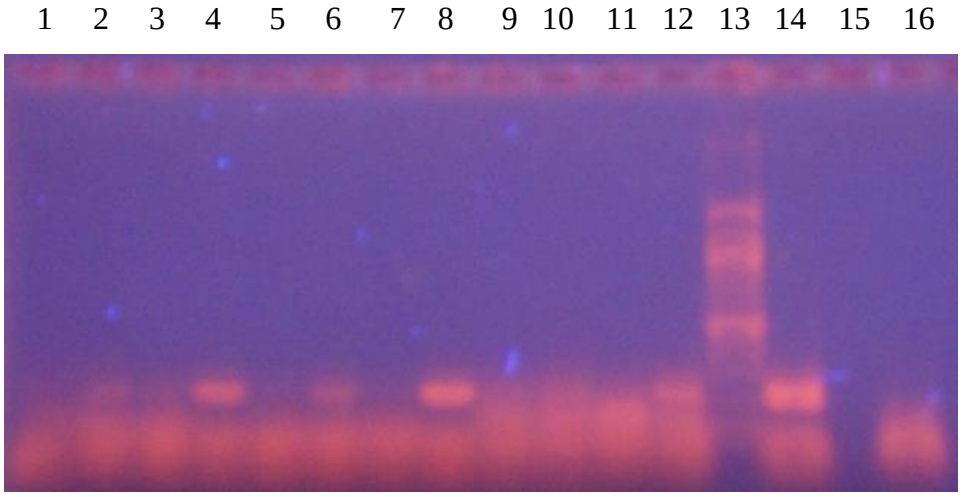

Fig. 1. Agarose gel electrophoresis of PCR products (250 bp) specific to Lactobacillus genus. 1 hr effect of lysosyme: 1. control buffer A, 2. buffer B, 3. immobilized lysozyme, 4. washing powder Amway; 3 hr effect of lysosyme: 5. control buffer A, 6. buffer B , 7. immobilized lysozyme, 8. washing powder Amway; 5 hr effect of lysosyme 9. control buffer A, 10. buffer B, 11. immobilized lysozyme, 12. washing powder Amway; 13. Standard 100 bp; 14. Positive control; 15. - ; 16. Negative control.

The optimal time of action of the lysing solution was determined: a) buffer B: 1 – 3 hr; b) immobilized lysozyme: 1 hr; c) 4% washing powder Amway: 3 hr. All lysis methods are suitable for use in molecular diagnostics using PCR.

4 Conclusions

The results presented in report show that all tested lysis methods are suitable for preparation of cell lysates.

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P20 QUANTUM DOT-BASED FÖRSTER RESONACE ENERGY TRANSFER BIOANALYSIS

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Summary
We describe a new configuration for Förster resonance energy transfer (FRET) between a quantum dot (QD) donor and a fluorescence dye acceptor attached to DNA molecule. This FRET sensor is aimed to be used in genomic analysis for DNA mutation detection applicable especially in cancer research.

1 Introduction

FRET is a process by which the energy of a fluorophore in excited state (the donor) is transferred through a non-radiative process to a second fluorophore (the acceptor) and then emitted at a longer wavelength. The intermediate non-radiative process is based on dipole-dipole interactions in the range of 1-10 nm [1].

Due to their unique photophysical properties (photostability, broad excitation band and emission band with very symmetric, narrow and tunable characteristic) QDs have many biological applications and are popular probe for optical detection [2]. Moreover, their high extinction coefficients make them ideal for transfer of large amounts of energy.

2 Experimental

We have synthesized a FRET probe consisting of CdTe QDs conjugated with oligonucleotide via zero-length crosslinking conjugation reaction between carboxylated QDs and aminated oligonucleotides. During the analysis, QDs probe serving as a donor was mixed with clinically-relevant PCR amplicons labelled with ROX (6-carboxyrhodamine) serving as an acceptor. The sample-probe hybridization was performed using a standard annealing protocol and followed by fluorescence measurement.

3 Results and Discussion

We used capillary electrophoresis with laser induced fluorescence detection (CE-LIF) and fluorescence spectroscopy for analyses of products of conjugation reaction and hybridization reaction. Electropherogram of products of conjugation reaction between

QDs and aminated oligonucleotide (Fig. 1) in ratio 1:1 shows the absence of peaks of free reactants (QDs) indicating complete conversion.

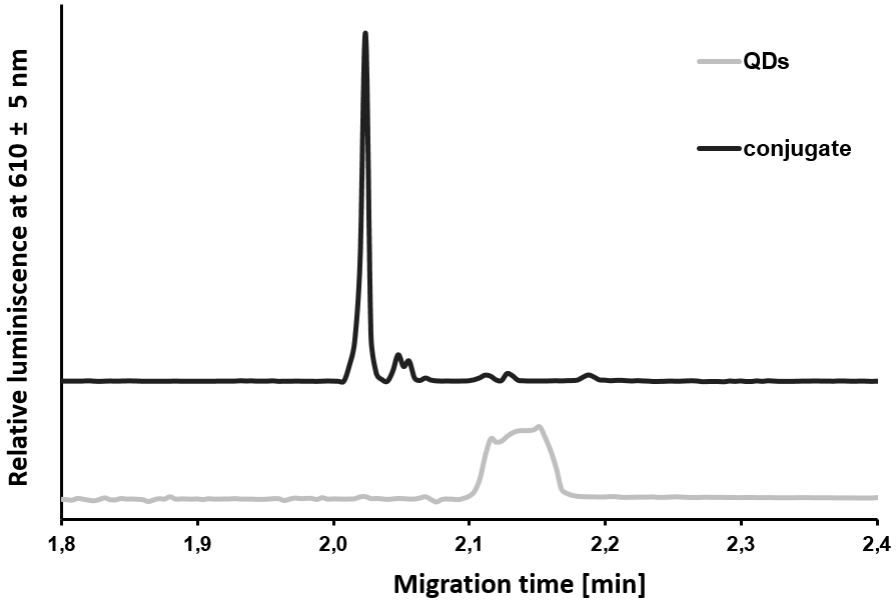

Fig. 1: Electropherogram of product of conjugation reaction.

Upon mutation-specific sample-probe hybridization, an increase in fluorescence intensity at 610 nm (the emission spectra maximum of ROX) confirmed a proper function of FRET at a presence of detected mutation.

4 Conclusions

We have presented new design and synthesis of the sensor based on FRET. Experimental data from capillary electrophoresis proved successful conjugation of QD and ssDNA molecule. The limit of detection evaluated for PCR amplified fragments of the sample was $5 \cdot 10^{-9}$ M.

Acknowledgement

This work was supported by Technology Agency of the Czech Republic (TA02010672), Grant Agency of the Czech Republic (project No. 14-28254S) and by institutional support RVO 68081715 of Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic, v.v.i.

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P21 MAGNETIC BEAD MEDIATED COMBINATORIAL CARBOHYDRATE SYNTHESIS

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Summary
Beads act as solid support in a wide variety of biomagnetic partitioning. Their size makes them particularly suitable for protein isolation for sample preparation from biological fluids, bioassays, selection of affinity binders, etc. Surface-reactive primary amino-groups allow immobilization of ligands through reductive amination of aldehyde or ketone groups without prior activation of the surface. For the time being, such beads have only been used in proteomics. The primary goal of this work was to develop new protocols for magnetic bead mediated combinatorial carbohydrate synthesis to be applied towards global glycomics research.

1 Introduction

N-glycosylation of proteins is ubiquitous on eukaryotic cell surfaces and in body fluids. The dynamically growing field of glycobiology/glycomics is devoted to defining structural and functional roles of glycans in numerous biological recognition processes, including, viral and bacterial infection, tumor metastasis, immune response and many other receptor-mediated signaling processes [1,2]. Chemical carbohydrate synthesis provides valuable means to produce glycans, which serve as model compounds to gain insight into *N*-glycoprotein structures and functions, as tools to study biomolecular interactions and as effectors to evoke biological responses [3]. However, due to the large number of possible linkage and positional isomers, chemical preparation of oligosaccharides is extremely time consuming and labor intensive in comparison to the synthesis of other biopolymers, such as peptides or nucleic acids. The synthesis of oligosaccharides can be characterized as the regio- and stereoselective formation of interglycosidic linkages. To date, there are no general applicable methods or strategies to synthesize large complex oligosaccharides and consequently the preparation of such compounds is consuming great challenge. One special feature on N-linked glycans is core and/or antennary fucosylation occurring in a large number on abnormal cell surfaces, such as cancer cells. Therefore the initial effort of the work presented here addresses a model for multi-fucosylated glycan synthesis by magnetic bead mediated combinatorial carbohydrate synthesis.

2 Experimental

SiMAG Amine beads (Chemicell, Berlin, Germany) were used to support the combinatorial sythesis reactions. Reductive amination was employed to bind the combinatorially synthetized components. The reaction products were monitored by means of protein binding from human serum. The binding was followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to visualize the results. For structural carbohydrate synthesis monitoring, APTS labeling and capillary electrophoresis with laser induced fluorescence (CE-LIF) detection were used [4].

3 Results and Discussion

Based on the experience of the last 30 years [5-9]. Our research program focuses on the preparation of a large number of closely related structural glycan determinants by means of conventional carbohydrate chemistry. Due to the high demand of generating a large variety of glycan structures, combinatorial carbohydrate synthesis was introduced. In this latter one, after the removal of the protecting groups the generated mixture of random oligosaccharides were evaluated by their interactions with human serum proteins.

The key compound of the synthetic route was fucosylated lactosamine**,** which can be considered as a selectively protected oligosaccharide structure. Random fucosylation of N-acetillactosamine acceptor was performed with galactose donor. The lactosamine possessed six free OH-groups, which all could take part in the reaction, possibly resulting in tri-, tetra-, pentasaccharides, etc. The donor molecule was added to the reaction mixture in such a ratio that mainly generated tetra-substituted forms.

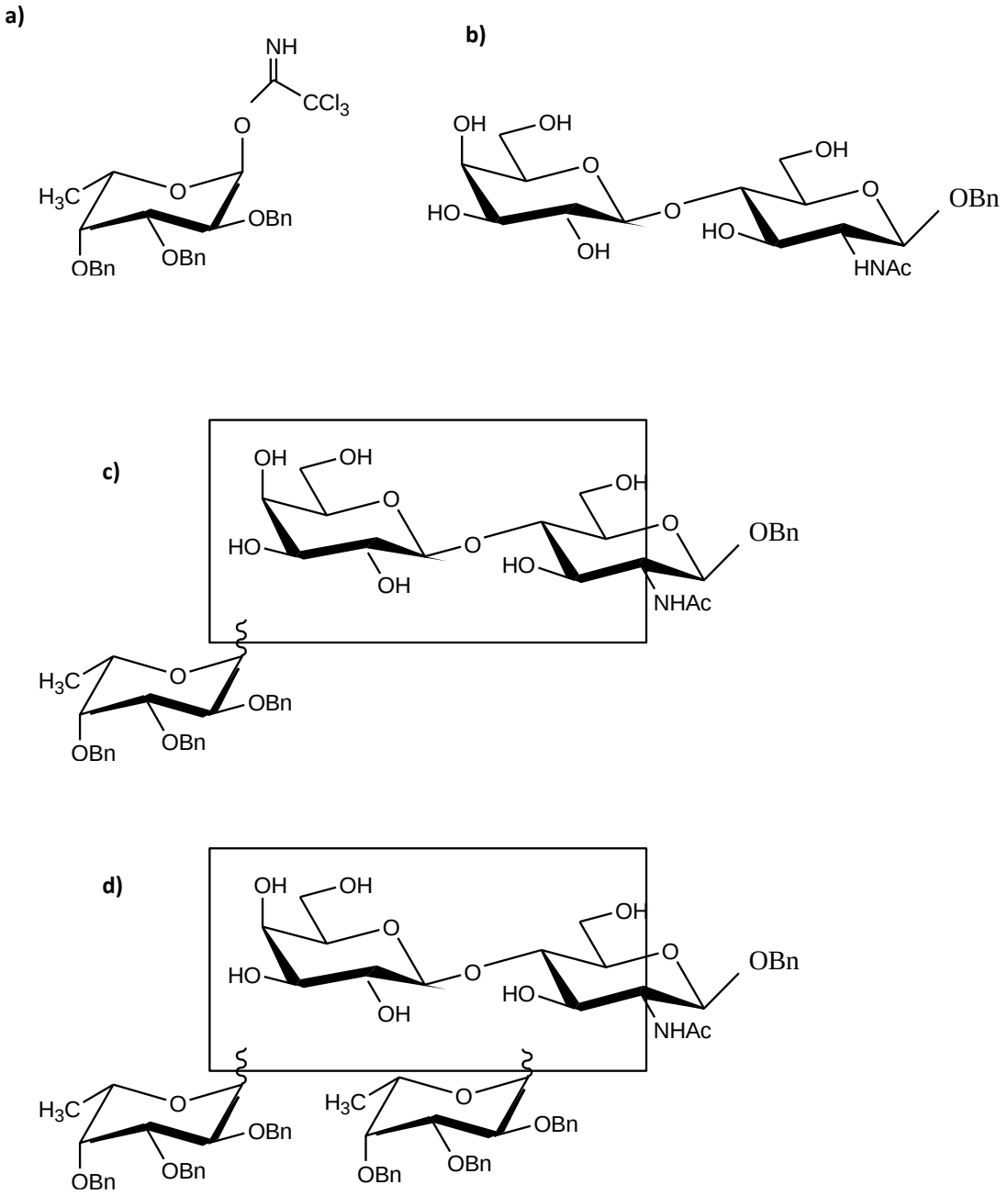

Fig. 1. Structural representation of the glycans involved in the combinatorial reactions. a) donor fucose, b) lactosamine acceptor c, and d) two of the possible products.

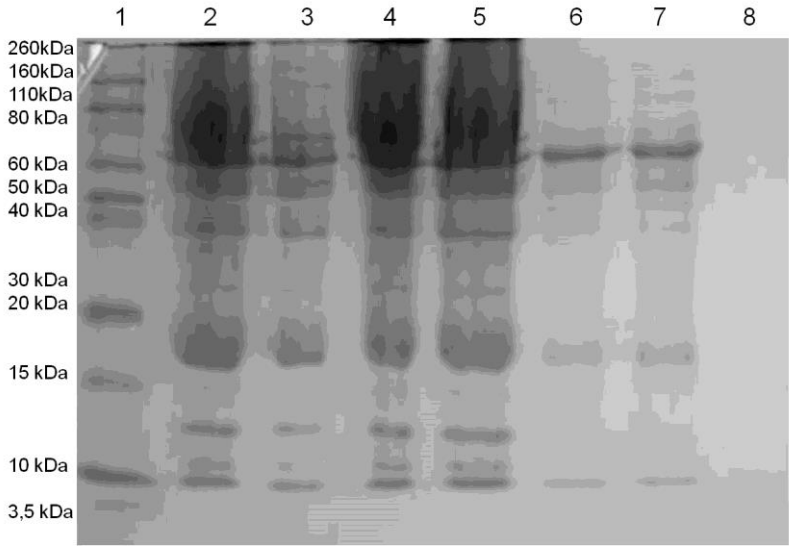

Fig. 2. Serum protein binding properties of combinatorial carbohydrate coated magnetic beads. Lane 1: protein ladder, Lane 2,3: human serum, Lane 4,5: non bound fraction, Lane 6,7: bound fraction, Lane 8: negative control.

Protein binding properties of the combinatorial synthesis reaction products were monitored by SDS-PAGE as shown in Figure 2. Lane 1 shows the protein ladder. Lane 2 and 3 are the control human serum samples. Lane 4 and 5 represent the non-bound fraction, which was washed off from the beads. The main results shown in lane 6 and 7 depicting that the combinatorially synthetized carbohydrate coated beads bound proteins in the 5 kDa, 15 kDa and 40-60 kDa size range. The last lane is a negative control. The combinatorially synthetized multi-fucosylated glycans bound to magnetic beads recognized specific sugar binding proteins form human serum, which will be identified by mass spectrometry in our future experiments.

4 Conclusions

Combination of classical and combinatorial carbohydrate chemistry seems to be a promising approach for the preparation of random oligosaccharide libraries for biochemical analysis (combinatorial). Glycobiology is devoted to define the structure/function relationship of glycans in numerous clinically significant biological processes. Identification of cancer-specific glycan structures can be useful in the development of new cancer therapies, for example, in generation of vaccines or antibodies targeting tumor-associated glycan antigens. Therefore, utilization of combinatorially synthesized carbohydrate structure-coated magnetic beads opens up new avenues for such investigations.

Acknowledgement

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P22 IDENTIFICATION OF HUMAN MICROBIOME IN HARD-TO-HEAL WOUNDS BY MALDI-TOF MS AND SEQUENCING

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Summary

This study is focused on the isolation, characterization and identification of the most abundant microorganisms in superficial wounds of patients (n = 50) suffering from bacterial infection. Primarily, the identification of the strains was carried out through Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) based on comparison of protein profiles (2-30 kDa) with database. Sanger sequencing of 16S bacterial gene was used for confirmation if the score was < 2.

1 Introduction

More than 25 years ago, mass spectrometry was identified as a potentially viable physical method for characterization of microbial samples on the basis of the detection of specific biomarker molecules [1]. The identification is based on differences in the observed ribosomal protein patterns, which form specific "fingerprint" of different organisms [2].

MALDI-TOF MS was exploited as an accurate and rapid identification tool, using the protein mass patterns, which are compared with patterns from a commercial Bruker Daltonics *database (*BDAL) of MALDI Biotyper software [3]. Due to a powerful software support, the method can be used for identification within few minutes, which is one of the advantages [4]. Moreover, sequencing of 16S rRNA gene (common marker for human microbiome studies [5]) was employed for the identification independent on analysis of protein patterns.

2 Experimental

2.1 Cohort of patients with bacterial infections

For evaluation, patients with surface or deep infection severity were selected. Total of 50 patients were enrolled into the clinical study with the age between 19 and 93 years, with majority (13 patients) in group a 70-79 years of age. 23 patients suffered from surface wounds and 27 from deep wounds.

2.2 MALDI-TOF MS identification of bacteria

The following extraction protocol was based on MALDI Biotyper 3.0 User Manual Revision 2, similar extraction method was used also in [4]. After extraction the samples were centrifuged at 14 000 × g for 2 min and 1 µL of the clear supernatant was spotted in duplicate onto the MALDI target and air-dried at room temperature. Then, each spot was overlaid with 1 µL of α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution (20 $mg.mL^{-1}$) in organic solvent (50 % acetonitrile and 2.5 % trifluoroacetic acid, both *v*/*v*) and air-dried completely prior to MALDI-TOF MS measurement on UltrafleXtreme MS (Bruker Daltonik GmbH, Bremen, Germany). Spectral data were taken in the *m/z* range from 2 000 Da to 30 000 Da, resulted from the accumulation of 240 laser shots targeted to different regions of the same sample spot. These data were analysed with the Flex Analysis software (Version 3.4).

2.3 DNA sequencing

Genomic DNA was isolated from lysed bacterial cultures *via* MagNA Pure Compact (Roche, Mannheim, Germany). For sequencing reaction the DTCS Quick Start Kit (Beckman Coulter, Pasadena, CA, USA) was used. To 20 µL sequencing reaction mixture, 98 ng of amplified fragment, 0.75 µL of 10 µM forward primer, 4 µL of DTCS Quick Start Master Mix and 1 µL of Sequencing Buffer were added. The purification of sequencing product was carried out using CleanSEQ kit (Beckman Coulter). Purified samples in Sample Loading Solution were transferred to the plate and DNA sequencing was performed using Genetic Analysis System CEQ 8000 (Beckman Coulter). Sequences were identified by comparison with NCBI database.

3 Results and Discussion

For the identification of bacterial entities we employed methods, which even though are complementary, are based on evaluation of completely different biomolecules - proteins and DNA [4]. When compared to Sanger sequencing, MALDI-TOF MS offers much shorter analyses time. By using this technique, wound microbiome may be discriminated within one hour from incubation, and thus this method is considered to form future trends in microbiome identification. Nevertheless, the classification is based on still developing database [6]; hence MALDI-TOF MS identification of non-databased bacteria has to be still connected with any other confirmation method. For this reason we firstly employed MALDI-TOF MS with condition: If score < 2.00 = 16S rRNA sequencing.

108 various bacterial samples were identified (35 of them had to be confirmed by sequencing) and confirmed strains were immediately databased to increase the future classification success. 23 different bacterial strains were identified, 17 various stains were detected at patients with deep wound and 16 different strains were found out at patients with surface wound (Fig. 1).

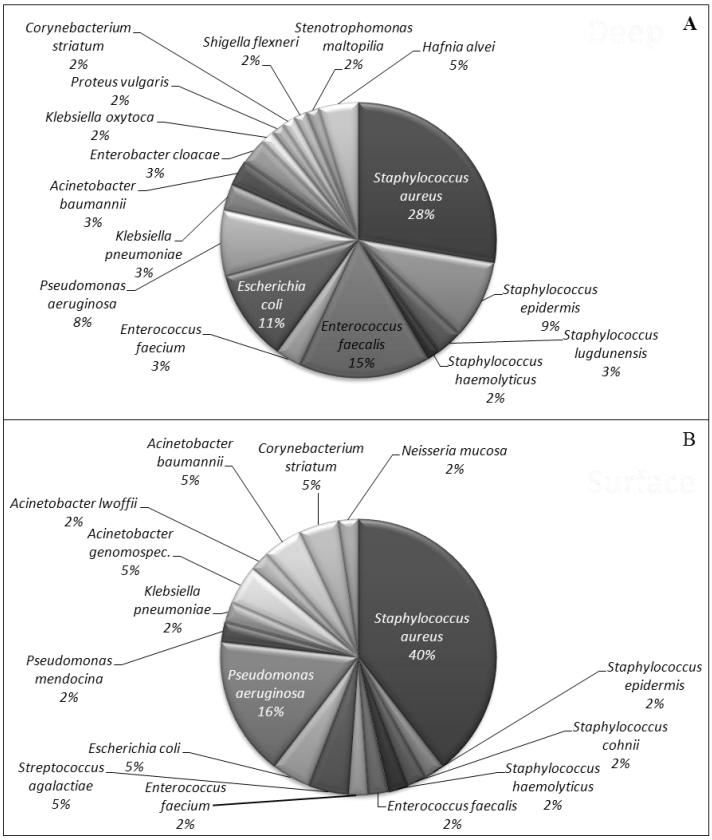

Fig. 1. Microorganisms composition in patients wounds with different severity – A) deep wounds, B) surface wounds.

As it is obvious from Fig. 1A, in the more serious infections (deep) the major role in composition of microbiome played *S. aureus* (28 % of identified strains) and *E. coli* (11 %). On the other hand, *E. coli* was not so often identified in surface

wounds group (5 % - Fig. 1B). Taken together, the microbiome composition in both groups exhibits substantial differences, and thus it can be hypothesized that presence of minority representatives as *Hafnia alvei*, *Proteus vulgaris*, *Staphylococcus lugdunensis* or *Enterobacter cloacae* in wound can significantly influence the infection severity.

4 Conclusions

The MALDI-TOF MS is very useful technique for rapid microorganism identification. The obtained data will be statistically processed by artificial neuronal network method and compared with patients' diagnoses for evaluation of connection between severity of infection and wound microbiome species composition.

Acknowledgement

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P23 NON-INVASIVE SAMPLING AND SUBSEQUENT CAPILLARY ELECTROPHORETIC ANALYSIS OF SWEAT AND SALIVA IN CLINICAL DIAGNOSIS OF CYSTIC FIBROSIS

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Summary
Capillary electrophoresis (CE) with double opposite end injection (DOEI) and contactless conductivity detection (C4D) was used in a novel approach for diagnosis of cystic fibrosis (CF). A simple, fast and inexpensive „skin wipe“ technique for sweat sampling was developed as a time-saving substitute for conventional, iontophoretic sweat sampling. Saliva sample was also obtained. A number of major target analytes (inorganic/organic anions and inorganic cations) were quantified simultaneously in these samples. By applying principal component analysis (PCA) to ion concentration ratios, rather than to individual ion concentrations, more accurate diagnostic tool for CF was obtained. We demonstrate that by using the developed approach and comparing a group of healthy individuals to patients diagnosed with CF, two clearly distinguished clusters can be observed without any data overlap. This approach may thus set a basis for a new method to diagnose CF more reliably.

1 Introduction

Cystic fibrosis is a lifelong, hereditary disease that causes thick, sticky mucus to form in the lungs, pancreas, and other organs. In the lungs, this mucus blocks the airways, causing lung damage, making it hard to breathe, and leading to serious lung infections. CF is caused by a mutation on transmembrane conductance regulator gene (CFTR) and results in defective ion transfer through the epithelial cellular membranes [1].

2 Experimental

2.1 Electrophoretic system

Analyses were performed using a purpose built CE instrument consisting of a power supply unit (DX250, EMCO USA), an in-house built C4D detector and an AD converter (Panther 1000, ECOM s.r.o., Czech Republic). The CE system is described in more detail in [2]. A fused-sillica separation capillary (50 µm ID, 375 µm OD, 50 cm total length) was used with BGE consisting of 20 mM 2-(N-morpholino)ethanesulfonic acid (MES), 20 mM L-histidine (HIS) and 2 mM 18-Crown-6. The separation voltage was +15 kV.

2.2 Sampling

Skin wipe samples were obtained from volunteers and patients with diagnosed cystic fibrosis by using a cotton swab (thoroughly pre-rinsed with DI water and dried) that was wetted with 100 µl of DI water. A defined area of skin (1 x 5 cm) was repeatedly (10x) wiped using a plastic template. The cotton swab was then immersed in 400 µl of DI water, let stand for 3 min to extract the analytes and discarded. The extract was analyzed by CE. Saliva samples were obtained from. All samples were stored at -20°C when not in use.

3 Results and Discussion

3.1 Analysis of skin wipe samples

An optimized separation electrolyte system was developed on the basis of previous research [2]. The electrolyte allows simultaneous determination of anions and cations by applying double opposite end injection (DOEI). Figure 1 shows an electropherogram of mixture of standards and a sweat sample.

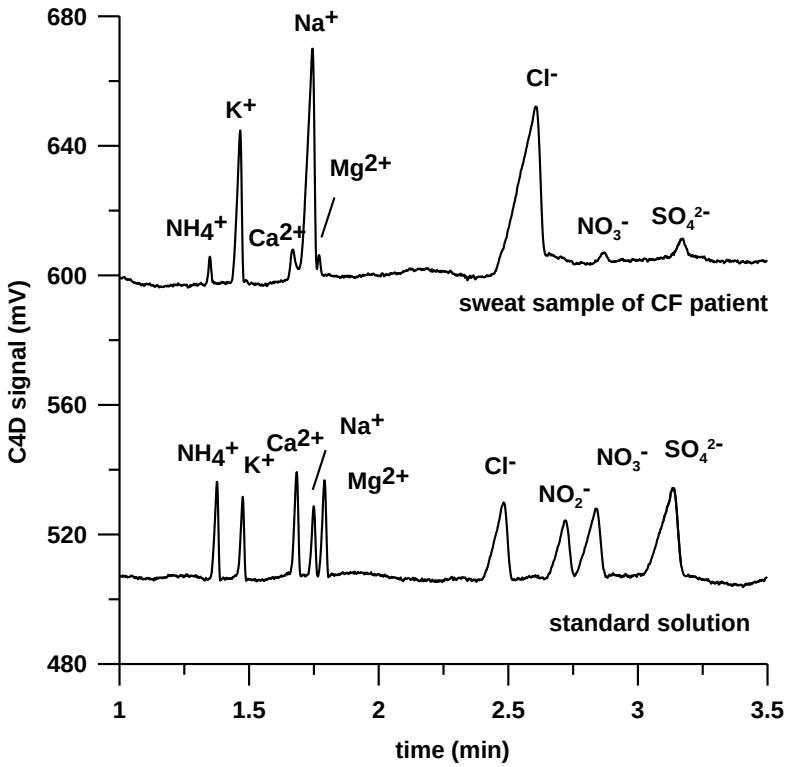

Fig. 1. DOEI-CE separation of a sweat sample of CF patient and a standard solution. CE conditions: Voltage 15 kV, C4D detection, HD injection of sample/BGE/sample: 20/20/20 s from the height of 10 cm. Ion concentrations in standard 50 µM each.

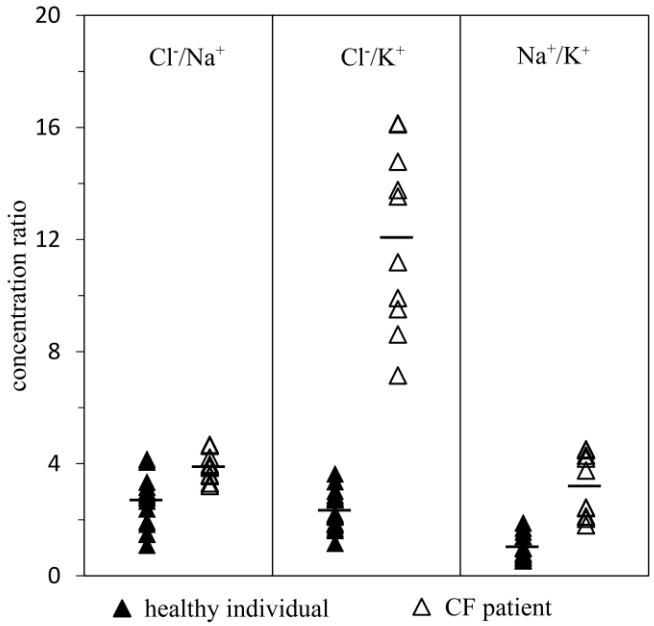

Fig. 2. The concentration ratios of chloride, sodium and potassium in a group of healthy individuals (under various conditions, as described in Figure 3.) and patients with cystic fibrosis. Samples are analyzed by DOEI-CE, CE conditions: the same as in Figure 1.

The skin wipe samples of healthy (H) and CF patients were compared and it was noted that although the chloride levels alone provided some clinical relevance, the concentration levels of H and CF overlapped in many cases. This applied for other ions as well. However, when ion ratios rather than individual ion concentrations were used, clearly Cl^-/K^+ or Na^+/K^+ gives much better differentiation between the healthy and CF (Figure 2).

3.2 Principal component analysis and CF diagnosis

The measured ion ratios from all experiments (healthy, robustness study, CF patients) were subjected to PCA. The potential of PCA is shown in Figure 3. By comparing a group of healthy individuals, healthy individuals at different times of a day (morning, noon and evening), healthy individuals after physical activity and healthy individuals whose forearms were precleaned with 70% ethanol to patients diagnosed with cystic fibrosis using PCA, two distinct clusters can be observed. These clearly distinguish CF patients from healthy individuals.

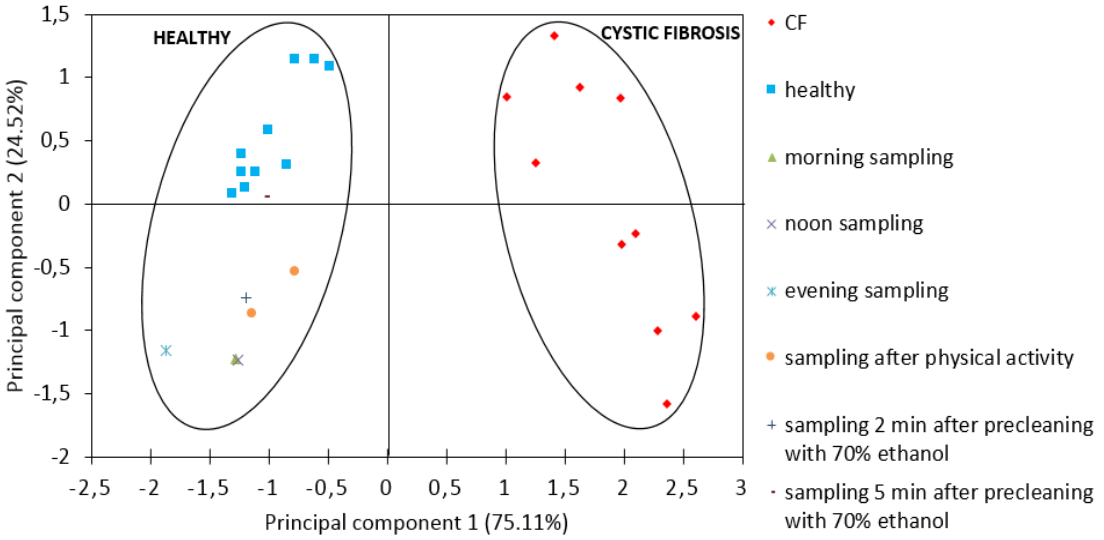

Fig. 3. Peak area ratios data from all samples analyzed by standard principal component analysis (PCA).

3.3 Analysis of saliva

In yet another preliminary experiment, we have analyzed the saliva from healthy and CF patients, and have found some remarkable differences in ion composition, in particular the SCN^-. The research is in progress and the results will be published soon. Figure 4. shows an example of three clinical saliva samples analyzed by our CE-DOEI-C4D system.

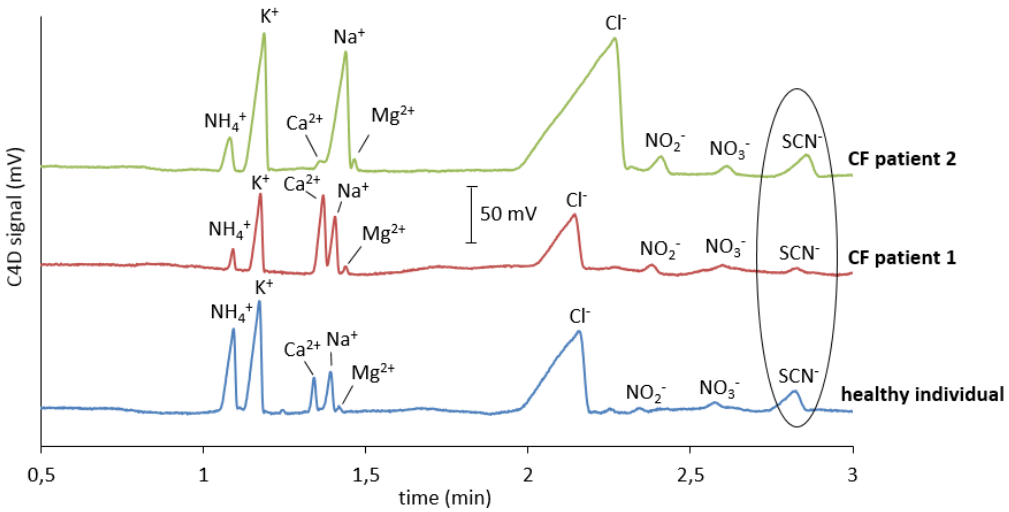

Fig. 4. DOEI-CE separation of saliva of healthy individual, CF patient 1 and CF patient 2. Samples are analyzed by DOEI-CE, CE conditions: the same as in Figure 1.

4 Conclusions

A new simple, fast and inexpensive „skin wipe“ technique for sampling of sweat from patient’s forearm was developed and might be suitable as a surrogate to conventional, time-consuming, iontophoretic sweat sampling and analysis. The developed method is also suitable for monitoring of salivary ionic content. CE-DOEI-C4D offers another

dimension in CF diagnosis - by simultaneously quantifying several ions and applying principal component analysis (PCA) to the ion ratios, more accurate diagnostic tool can be obtained.

Acknowledgement
The authors acknowledge the financial support from the Grant Agency of the Czech Republic (Grant No. P206/13/21919S). Part of the work was realized in CEITEC - Central European Institute of Technology with research infrastructure supported by the project CZ.1.05/1.1.00/02.0068 financed from European Regional Development Fund.

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P24 QUANTUM DOT-BASED LABELING PROCEDURES FOR PREPARATION OF SPECIFIC ANTIBODY CONJUGATES

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Summary
Due to routine commercial unavailability of quantum dot-labelled antibodies, which could serve as highly sensitive secondary antibody in ELISA-based immunosensors of e.g. biomarkers, the main goal of our work was to develop efficient and as simple as possible method of antibody conjugation with quantum dots (QDs). For this purpose we applied two different approaches. Developed methods are based on carbodiimide technique of covalent coupling or on utilization of silica mesoporous nanoparticles as QDs carrier. Formed conjugates are detected by square wave voltammetry (SWV) with miniaturized screen-printed sensors (SPE) and supplemented by polyacrylamide gel electrophoresis in Tris-borate-EDTA buffer (TBE-PAGE) as fast in-between confirmatory technique.

1 Introduction

Due to unique optical and electrochemical properties of QDs (especially CdSe core ZnS capped designed) they represent widely-spread smart type of nanomaterial frequently used for conjugation with various types of biorecognition molecules [1-4]. For our purpose we utilize QDs as antibody label in electrochemical ELISA-based immunosensors, which are used for analysis of tumor markers in early stage of disease (ideally before the first clinical symptoms) where commonly used analytical methods have sensitivity limitations.

Commonly used methods for QDs labeling of antibodies principally consist in attachment of one QD could significantly increase the electrochemical response and thereby also final sensitivity.

We introduce two protocols for preparation of target both conjugates based on carbodiimide coupling. First approach uses biofunctionalized magnetic microparticles as anchor for following labeling by QDs carried out through created immunocomplex on its surface. The second protocol uses nonmagnetic mesoporous amino-functionalized silica nanoparticles firstly wrapped with QDs and then this tag is used for conjugation with antibodies. Quality and functionality of final conjugates created by both methods was verified electrochemically by square wave voltammetry (SWV) with SPE. Moreover TBE-PAGE with UV visualization was included as in-between verification method. We chose apolipoprotein E (ApoE) and anti-apolipoprotein E antibodies (anti-ApoE) as a model system.

2 Experimental

2.1 Biofunctionalized magnetic carrier with immunocomplex for conjugation with QDs

ApoE (BioVision Inc., Milpitas, California, USA) in amount appropriate to creation of monolayer (in our case 25 µg) was immobilized to 1 mg of SiMAG-carboxyl particles (1 µm, Chemicell GmbH, Berlin, Germany) through two-step protocol using the standard carbodiimide technique modified to our purpose [5]. Biofunctionalized magnetic carrier was blocked by 0.1 M ethanolamine and treated immunosorbent was used for specific creation of immunocomplex on its surface by use of 25 µg of rabbit polyclonal anti-ApoE (Moravian Biotechnology, Brno, Czech Republic). Thereafter 0.5 mg of particles with immunocomplex underwent 10 minutes of activation in presence of 0.5 mg EDC in total volume of 500 µl. Overnight conjugation with 8 µl of 8 µM QDs (Qdot® 565 ITK™ carboxyl quantum dots made of CdSe/ZnS, Invitrogen, USA) in presence of 0.1 M phosphate buffer pH 7.3 at 4°C was followed. Finally effective elution of labelled antibodies was realized by use of 0.05% Trifluoroacetic acid (TFA) containing 0.5% SDS.

2.2 NH_2-functionalized silica nanoparticles with QDs for binding with antibodies

50 µg of mesoporous silica nanoparticles with amine functional groups (200 nm, Sigma-Aldrich, St. Louis, MO, USA) were used for conjugation with 10 µl of 8 µM QDs solution (Qdot® 565 ITK™ carboxyl quantum dots made of CdSe/ZnS,

Invitrogen, USA). Conjugation was carried out in presence of 0.1 mg of EDC and sulfo-NHS in ratio of 6:1 (w/w). Mixture of nanoparticles, QDs and cross-linkers was overnight incubated at 4°C with gentle mixing. Then washing by 0.1 M phosphate buffer pH 7.3 and PBS buffer pH 7.4 was carried out and 100 µg of this tag was conjugated with 15 µg of rabbit polyclonal anti-ApoE antibodies (Moravian Biotechnology, Brno, Czech Republic) again in presence of EDC and sulfo-NHS in the ratio mentioned previously. Overnight binding of specific antibodies at 4°C followed. Formed conjugates were washed by PBS buffer pH 7.4 and stored in the same buffer enriched by 0.05% TWEEN.

3 Results and Discussion

Both methods are based on using of EDC combined with sulfo-NHS as cross-linker reagents. These approaches were chosen due to simple but highly effective covalent binding. Although it is random immobilization procedure, we assume that due to the size of QDs, the binding site of antibody in not affected by QDs. The protocol based on magnetic particles as a solid phase consists of several steps which make it more time consuming experiment. On the other hand this method layout allows better manipulation and separation of target conjugate from unbound fraction of antibodies and QDs. What is more each step is controlled and final elution step provides us conjugates with QDs situated on the Fc fragment of labelled antibody and free binding site which is essential for the next immunospecific reaction. Compared to the approach mentioned above the protocol based on mesoporous silica nanoparticles conjugated with QDs as a special tag is preferable for routinely creation of quantum dot-based conjugates we had to face to negative effect of nonspecific sorption to the microtube surface during the whole experiment. Suitable option of reaction conditions was confirmed by electrochemical detection which demonstrated the functionality of created conjugates by both ways.

4 Conclusions

We used two different procedures for creation quantum dot-based antibody conjugates. Both protocols are functional and simultaneously are not accompanied by more complication. Essentially main goal was achieved. We were able to prepare conjugates of required specificity suited for our immunosensor. Further step would be improvement of sensitivity provided by our “home-made conjugates”.

Acknowledgement

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P25 ANALYSIS OF SACCHARIDES IN GRAPES AND WINE USING HPLC WITH VARIOUS STATIONARY PHASES

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Summary
The quality of wine is influenced by its saccharides content, especially glucose and fructose. Monitoring of wine oligosaccharides is of great interest, because of their physiological, medical and food importance. The aim of study was to develop method for fast analysis of saccharide composition of wine, grape juice and grapes.

1 Introduction

The wine quality, character and taste is strongly influenced by saccharide part, mainly by glucose and fructose. Additionally, other monosaccharides (e.g. mannose, galactose, xylose, arabinose, ribose a rhamnose), oligosacharides (e.g. lactose, saccharose, maltose, trehalose), and sugar alcohols (e.g. mannitol a ribitol) can be also detected[1]. Qualitative and quantitative representation of saccharides significantly changes during fermentation process[1,2]. Because of the importance of saccharides from medical and food point of view, this study is focused on the development of method for fast monitoring of individual sugar components in wine. For this purpose, we used combination of techniques based on sample isolation (solid phase extraction – SPE), separation (high pressure liquid chromatography – HPLC) and detection (refractive index, UV, mass spectrometry).

2 Experimental

2.1 Sample preparation

Standard solutions of mono- and oligosaccharides were prepared in individual mobile phases. Grape juice and wine samples originated from wine region Morava.

2.2 Solid phase extraction (SPE)

SPE sample purification process was performed using lab-made equipment and for individual experiments following SPE columns were used:

Strata XL	Phenomenex
Bond Elut C18 EWP	Agilent
Strata C18 EWP	Phenomenex
Strata SDB-L	Phenomenex
Discovery DSC-PH	Supelco
Discovery DPA-6S	Supelco
SOLA HRP	Thermo
SampliQ Carbon	Agilent
Supelclean ENVI-CHROM P	Supelco
Isolute NH_2	Biotage
Bond Elut Certify II	Agilent
StratoSphere PL-Mixed	Agilent
Chromabond HR-XA	Macherey Nagel
Discovery DSC-SAX	Supelco

2.3 High pressure liquid chromatography (HPLC)

HPLC analyses were performed on HP 1100 series (Agilent) connected to refractometric (RID), diode array (DAD) and mass spectrometric (MSD) detectors. Following chromatographic columns were used for samples separations: Hypercarb (150×2.1 mm, 3 μm; Thermo), Kinetex HILIC (150×2.1 mm, 2.6 μm; Phenomenex), Prevail Carbohydrate ES (150×2 mm, 5 μm; Grace), TSKgel Amide-80 (150×2 mm, 3 μm; Tosoh), ZIC-HILIC (150×2.1 mm, 5 μm; Merck) at a flow rate of 0.2 mL/min.

2.4 Mass spectrometry

MS and MS^n spectra were acquired using ESI mass spectrometer (equipped with ion trap) Esquire LC (Bruker Daltonics). Mass spectra were recorded in both the positive and negative ion mode.

3 Results and Discussion

In this work, we compared and optimized purification steps for sufficient removing of interfering compounds within wine samples. As SPE sorbents, we tested hilic and reverse stationary phases and for HPLC separation we used various stationary phases (zwitterion, silica gel, amine, amide or graphite phase).

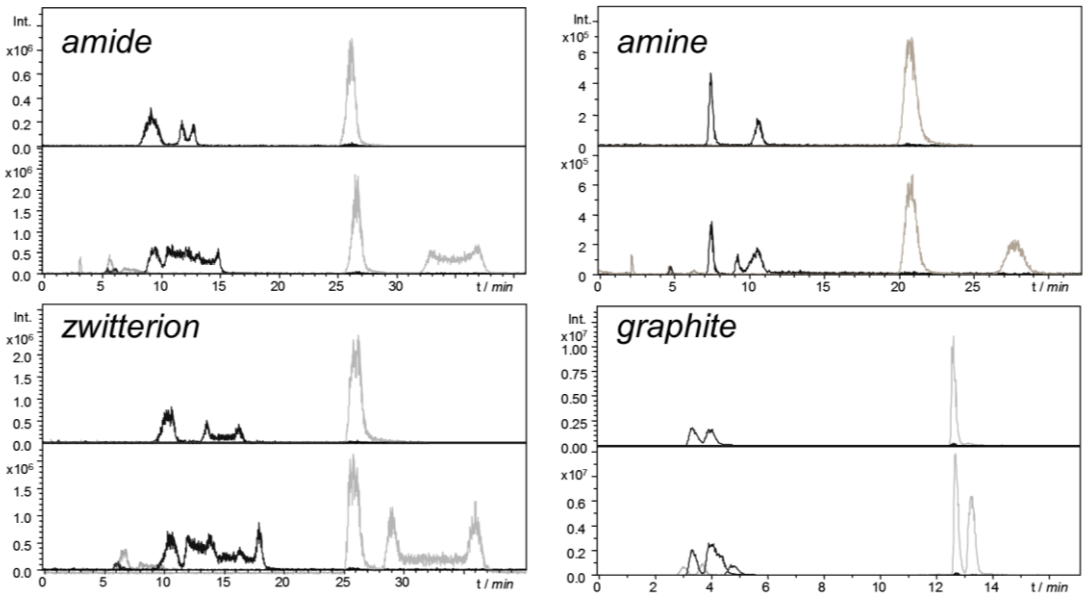

Fig.1. Separation of model mixture of saccharides (Fru, Glc a Sac) - comparison of individual columns.

Optimized procedure is based on connection of SPE on mixed-mode anion exchanger, separation on amino column (gradient of 85 - 60 % of acetonitrile results in sufficient resolution of analysed saccharides with no anomer resolution of reducing sugars) with sensitive ESI MS detection in both ion modes.

Separation of mono- and oligosaccharides and oligosaccharides concentration was performed using graphite carbon-based SPE.

Determined content of glucose and fructose in wine samples originated from Morava region was 0.35–1.33 mg/mL and 0.17–7.24 mg/mL, respectively. The ration of both mentioned saccharides strongly differed for each sample: 0.40–8.77. Other saccharides detected in our wine samples were ribose and rhamnose, only.

4 Conclusions

Method for analysis of saccharide content in grape juice and wine was developed in this study. The method is based on sample preparation using non-retentive SPE with mixed mode phase (reverse and anion exchange) and subsequent HPLC-MS analysis. The method provides sufficient resolution of separated saccharides. Moreover, anomer resolution was not observed. Using of narrow-bore column and MS detection in positive ion mode provided the best results.

Acknowledgement

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P26 THE STUDY OF INFLAMMATORY MARKERS OF ONCOLOGICAL PATIENTS BY MULTIVARIATE DATA ANALYSIS

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1 Introduction

Computer data handling of the results from laboratory tests creates new opportunities to support the process of diagnosis in laboratory medicine and clinical chemistry. This is an area of laboratory measurements that has brought new elements to the theory of analytical chemistry. Multivariate Data Analysis (MDA) is interdisciplinary branch between chemistry, mathematics and computer science, which deals with an exploratory analysis of the measured data, the classification of measured objects or multivariate calibration [1]. It provides a guideline for chemoinformatics, the way from data to knowledge. More widespread and more used chemometrics tools allow to reveal important relations and information in the data, as well as to obtain the data necessary to carry out the decisions, that solve the problem. All outlined statistical methods of MDA applied in data processing to the results from biochemical tests in clinical practice can be generally used to the evaluation of any analytical measurements [2, 3].

2 Experimental

2.1 Clinical determination

The analytically determined biochemical parameters used in the study are: the oncomarker CSI (carcinoma serum index), the inflammation markers CRP (C-reactive protein) and PCT (procalcitonine) as well as further, routine laboratory parameters resolved in the serum. Altogether 300 samples were gained from 106 patients and were analyzed by a fully automated biochemical analyzer Cobas 6000, Roche Diagnostics, Mannheim, Deutschland, and by hematological analyzer CELLTAC F, Nihon Kohden Corporation, Tokyo, Japan.

2.2 Statistical evaluation

Principal component analysis and cluster analysis belong to the unsupervised methods, because there is no supervisor in the sense of known membership of objects to classes. Classification methods - linear and quadratic discriminant analysis are supervised methods because the membership of objects to particular cluster is known in advance. All mentioned methods were used for assess the interaction of some markers of inflammation (CRP, Procalcitonin, Leukocyte …) in cancer patients and the grade of malignancies, that were evaluated according to carcinoma serum index (ratio of Orosomucoid and Prealbumin).

For the study of inflammatory markers of oncological patients by MDA following statistical software packages were used: Statgraphics Plus Professional 16.0, Systat 12.0, SPSS 15 and Microsoft Excel 2010.

3 Results and Discussion

Studied data set consists of 268 cancer patients, 168 men and 100 women who were depending on the extent and severity of disease hospitalized at National Oncological Institute in Bratislava, Department of Surgical Oncology, Internal Medicine Department, Transplantation Unit and Department of Anesthesia and Intensive Care.
Diagnostic efficiency was evaluated by means of MDA methods and mutual correlations between observed parameters in patients and suspected illness was specified. For the training data sets and for the validation sets the classification of patients into classes according to extent of disease had good span values (67 - 94 %) for men and women category. After reduction to six variables (Thromb, Prea, Ca, Na, K and Hgb) the most prosperous model with a success rate of 96.0% for classification and cross-validation of 89.0% was obtained by quadratic discriminant analysis for women dataset.

4 Conclusions

The objective of the chemometrical data processing, applying mathematical and statistical techniques realized by means of modern software packages, was to discover information, hidden in the measured data series, in order to better understanding of inflammatory processes of oncological patients.

The developed multidimensional models based on inflammatory markers enable to predict the category, to which the given patient's sample belongs which help in patient's treatment.

Acknowledgement

Thank you to all who participated in this study.

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P27 EFFECT OF DIFFERENT SEPARATION MATRIX COMPOSITION ON SMALL NUCLEIC ACIDS SEPARATION BY COMBINATION OF CAPILLARY IZOTACHOPHORESIS AND CAPILLARY ZONE ELECTROPHORESIS

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1 Introduction

Analysis of ions and substrates in biological material was followed by a great development of protein and enzyme diagnostics more than two decades before. Now, the nucleic acids (DNA and RNA) are commonly applied in diagnostics and therapeutics in human and veterinary medicine. The basic instrument in analysis of nucleic acid is a hybridization of two complementary single-stranded sequences of nucleic acid. Today it is possible to measure several copies of the nucleic acid by amplification techniques, for example by PCR in picomoles and attomoles level. But the nucleic acid diagnostics requires a new way of thinking and more sensitive and faster methods, especially in medical area [1]. Since 1993 several groups have developed new nucleic acid separation and quantification techniques based on capillary electrophoresis (CE) techniques, especially capillary isotachophoresis (ITP) [2]. The ITP is modern amplification-free analytical technique, offers subnanomolar sensitivity, cheap, robust and simple-mode operation, short time analysis and huge preconcentration of analytes. Under an electric field, ions of analytes are focused and simultaneously separated into segregated bands according to their effective electrophoretic mobilities. We are proposing a combination of ITP with capillary zone electrophoresis (CZE) to the analysis of small fragments of nucleic acid by principle hybridization target with the oligonucleotides detection probe by UV-VIS detection. This technique has found widespread, routine application, including environmental monitoring, food analysis, biological detection, though none of this report investigated the sieving matrix on the mobility of DNA oligonucleotides with the same length of sequence under denaturation and hybridization conditions using non-microfluidic ITP-CZE. We present ITP-CZE combination with active cooling of capillaries and use different separation matrixes following the previously published method [3].

2 Experimental

2.1 Oligonucleotides and Reagents

For hybridization study, we used fully complementary DNA oligonucleotides (target-probe) with no secondary structure (oligo 8 - 5´ d(TCT CTT CCT TC) 3´ and oligo 9-5´ d(GAA GGA AGA GA) 3´). To obtain the DNA duplex conformation, the oligonucleotides were mixed in water at equimolar rations in concentration range from 1 pM to 1 µM. The mixture before sampling was heated at 95 °C for 5 minutes for denaturation condition analysis or at 32 °C for 5 minutes for hybridization condition analysis. Synthesized oligonucleotides were stored at -20°C in nuclease free deionized water.

2.2 ITP-CZE analysis of oligonucleotides

A Villa Labeco ITP-CZE (Slovakia) with 180 mm long, and 300 µm inner diameter separation capillary with double-jacket water cooling (4 °C), and with UV-VIS detector (260 nm) was used. Measurements of ITP-CZE parameters were performed under a 100µA constant current with used 2-amino-2-hydroxymethyl-propane-1,3-diol based electrolytes [2] and 0,1% hydroxyethyl cellulose (HEC) for suppression of electroosmotic flow in a leading electrolyte (LE) and three different sieving matrix (2% dextran, 2% polyacrylaamide - PAA, and 0,6% polyethylenimine - PEI) in a background electrolyte (BGE). All chemicals were obtained from Sigma-Aldrich, and all solutions were prepared in ultrapure Millipore water, and stored at 4°C.

3 Results and Discussion

We experimentally demonstrate the hybridization and separation models for on-line combinations, non on-chip ITP-CZE with used the model DNA complementary oligonucleotides in different separation matrix and temperature conditions.

We are able to separate complementary DNA oligonucleotides with an identical length, but the different base composition. The addition of different sieving matrix (Fig. 1) to GBE had an effect on the separation of single-stranded (ss) and double-stranded (ds) oligonucleotides forms. However, the hybridization of nucleic acid is a dynamic process, the hybridization intermediate between single and double bonds was observed as shown in Fig. 1. One of the most critical hybridization conditions is the temperature of hybridization which was regulated by active cooling of capillaries. Biological samples nucleic acid hybridization assay with better regulation and monitoring of hybridization dynamics, are in progress.

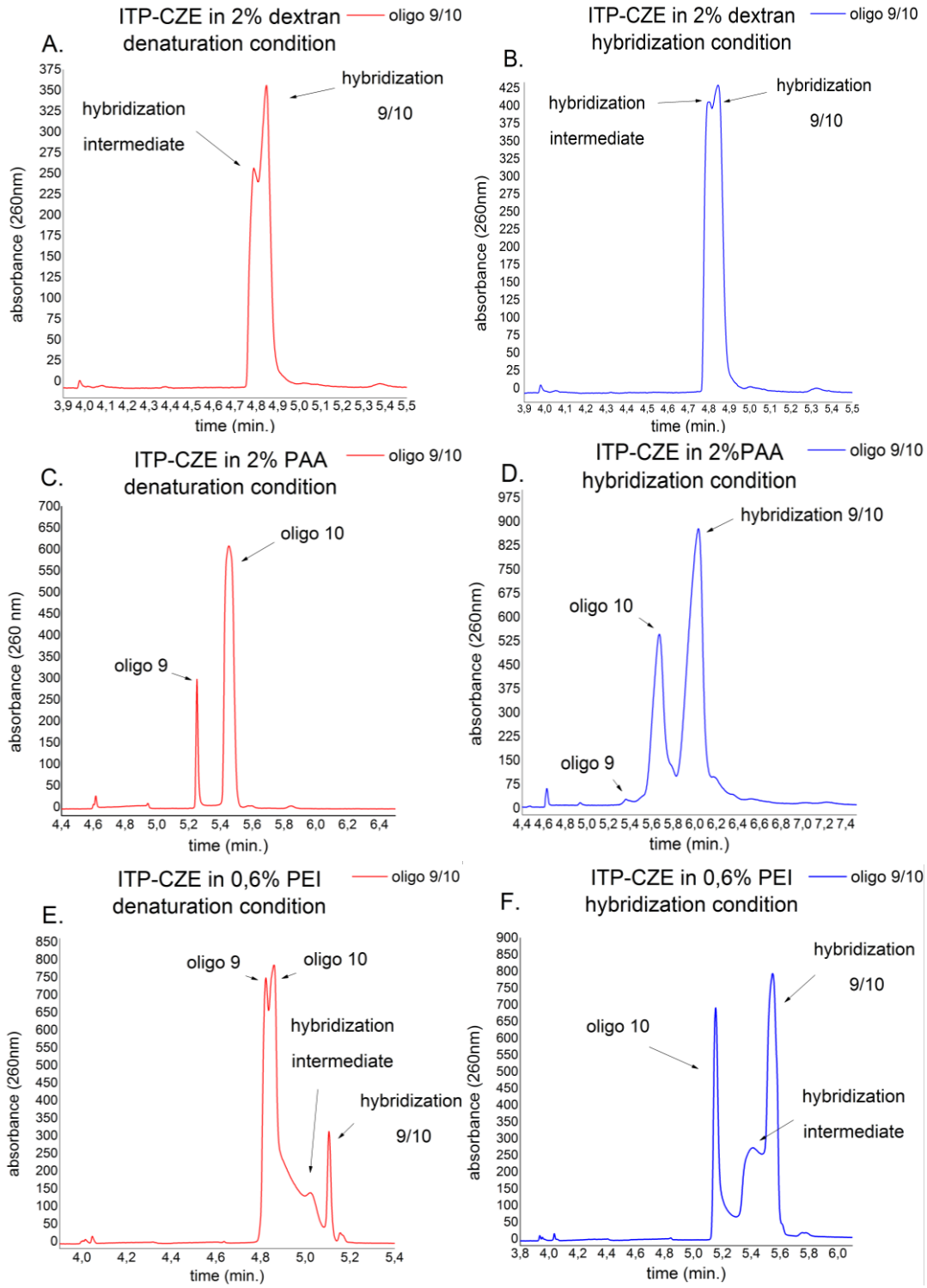

Fig. 1. Initial demonstration of the ITP–CZE separations and hybridization assay of model complementary oligonucleotides (oligo 8 & oligo 9) under denaturation and hybridization temperature conditions with addition of different sieving matrix to the electrolytes: (A;B) 2% dextran, (C;D) 2% polyacrylaamide (PAA), (E;F) 0,6% polyethylenimine (PEI). UV-VIS detection at λ = 260 nm.

4 Conclusions

This paper demonstrated the initial study of analyzing fully complementary short DNA oligonucleotides with ITP-CZE. We were able to concentrate DNA oligonucleotides with same length based on different effective mobility in ITP mode only with 0,1% HEC in leading electrolyte and subsequently separate this oligonucleotides in CZE mode with different common isotachophoresis sieving matrix. It was found, for visualization separation and hybridization results were most suitable 2% linear acrylamide. Total methods time was less than 20 minutes with the limit of detection (LOD) of 15 pM. Our future research activities include performing ITP-CZE clinical applications for miRNA cancer diagnostic assay.

Acknowledgement

This work was carried out with the financial support from the VEGA grants Nos. 1/0962/12, 1/1305/12 and Comenius University Grant No. UK/389/2014.

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P28 THE USE OF IONT POTENCIAL AT THE BACTERIAL DNA SEPARATION BY AMINO FUNCIONALISED MAGNETIC NANOPARTICLES

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Summary

Magnetic nanoparticles modified with poly (L-lysine) - polylysine (F79_L3) were used for magnetic separation of bacterial DNA from dairy products. The DNA concentration was determined by UV/VIS spectrophotometer. The presence of the target DNA was verified by PCR.

1 Introduction

The lactic acid bacteria are natural part of the gastrointestinal tract of animals and also people. They are also the part of many different kinds of food and dairy products. Polymerase chain reaction (PCR) has become a powerful diagnostic tool for the identification of beneficial microorganisms (probiotics) in foods. A high quality of isolated DNA is necessary for DNA amplification by PCR. As a real food matrix it has been used white yogurts, liquid dairy products, and flavored yogurts.

2 Experimental

At start, 1 ml of the overnight grown cell cultures was used for preparation of cell lysates. After a centrifugation (10 000 g), the pellet was resuspended in 500 µl of lysis buffer B (10 mM Tris pH 7,8; 5 mM EDTA pH 8,0; 3mg/ml lysozyme). Then, SDS (0.5 %) and proteinase K (1 µg/ml) were added in some cases and the mixture was incubated at 55 °C for 1 day.

In the next step the mixture for the DNA isolation was prepared: 400 µl phosphate buffer pH 7, 8, 400 µl crude cell lysate and 100 µl magnetic nanoparticles covered by *poly* (L-*lysine*) – polyLYSINE (F79_L3) (mw = 93800) (2 mg/ml) resuspended in the phosphate buffer. Microspheres were prepared by D. Horák from Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic. The mixture was incubated at room temperature (1 hour). Then, the magnetic microspheres were twice washed by 70% ethanol. DNA was eluted into TE buffer (100 µl, pH 9.0).

The amount and quality of isolated DNA was estimated using UV spectrophotometry (UV/Vis NanoPhotometer - Implen, Germany). The quality of isolated DNA was checked by the PCR method, using the *Lactobacillus* genus specific primers [1]. The PCR products were detected using agarose gel electrophoresis (1.8 %, 80 V, 2 hours).

3 Results and Discussion

The PCR products were detected using agarose gel electrophoresis (1.8 %)., 80 V, 2 hour) - Fig. 1.

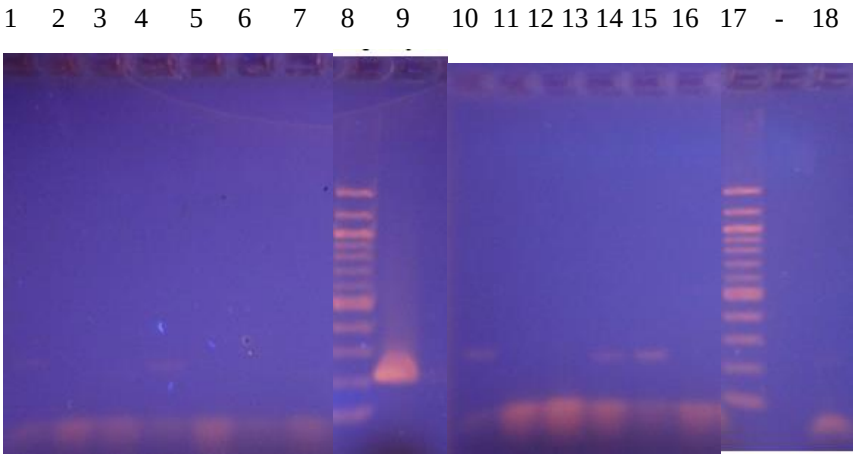

Fig. 1. Detection of PCR product – Lactobacillus genus. 1 µl DNA in PCR mixture 1 µl: 10x diluated DNA: 1. Smetanový bílý jogurt Kunín, 2. Klasik bílý jogurt OLMA, 3. Actimel bílý, 4. Acidofilní mléko, 5. Smetanový jogurt Florian – jahoda, 6. Smetanový jogurt jahoda z Valašska, 7. Naše BIO jogurt jahodový OLMA; 8. Standard 100 bp; 9. Pozitive control; nondiluated: DNA: 10. Smetanový bílý jogurt Kunín, 11. Klasik bílý jogurt OLMA, 12. Actimel bílý, 13. Acidofilní mléko, 14. Smetanový jogurt Florian – jahoda, 15. Smetanový jogurt jahoda z Valašska, 16. Naše BIO jogurt jahodový OLMA; 17. Standard 100 bp; 18. Negative control.

4 Conclusions

DNA was isolated from dairy products by magnetic microspheres polyLYSINE (F79_L3) at a quality suitable for the PCR. Magnetic separation of the bacterial DNA is a simple, quick isolation method. This method does not require the use of organic toxic substances.

Acknowledgement

The financial support of internal grant FCH-S-14-2325 is gratefully acknowledged.

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P29 DETERMINATION OF TOTAL APIGENIN IN EXTRACTS OF *PETROSELINUM CRISPUM*

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Summary

A micellar electrokinetic chromatography method was developed for the determination of apigenin in the Parsley leaves (*Petroselinum crispum)*. A buffer solution of 30 mM sodium borate (pH 10.2), 10% acetonitrile and 10 mM sodium dodecyl sulfate was found to be the most suitable BGE for this separation. The method was validated and calibrated in the range 56–225 mg/g (r^2=0.9890). The limits of detection and quantification were 0.5 µM and 1.0 µM, respectively, and the average recovery was 97.8%. The effects of pH, surfactant concentration and organic modifier on migration time, resolution and peak shape were also studied. The content of apigenin in the extracts from leaves of *P. crispum* was successfully determined within 12 minutes.

1 Introduction

A large number of studies suggest that a high intake of vegetables and fruits in diet can significantly reduce the risk of cardiovascular diseases and cancer. Bioactive components are compounds which occur in small amounts in food of vegetable origin [1]. One group of bioactive compounds, with a significant influence on human health are flavonoids, which are present in many commonly consumed fruits, vegetables and cereal products. Apigenin is a naturally occurring plant flavone. It is a potent antioxidant that exhibits anti-inflammatory activities, it is a scavenger of free radicals,

prevents LDL oxidation, atherosclerosis caused by the oxy-LDL and prevents oxidation of vitamins C, E and glutathione. Apigenin has some antitumor effect by inhibiting the tyrosine kinase activity of topoisomerase, angiogenesis and also protecting against oxidative damage to DNA [1-3].

Several methods have been established to determine some flavonoids using thin layer chromatography and high performance liquid chromatography [4,5]. However, owing to high resolving power, small sample volume, low reagent consumption and short analysis time, CE has been a very attractive research area in separation science. Among the works focused on flavonoids with strong pharmaceutical properties, determination of apigenin by CE has been reported several times [6]. We developed a MEKC method for identification and determination of apigenin in Parsley leaves.

2 Experimental

2.1 Apparatus and separation conditions

All experiments were conducted using Hewlett Packard HP^{3D} Capillary Electrophoresis System with diode array detector set at 390 nm. The fused-silica capillary with total length of 60 cm and effective length of 51.5 cm (50 µm I.D.) was used. The temperature of capillary cartridge was maintained at 25°C. The capillary was conditioned daily by washing with 0.1 M NaOH (5min), water (5 min) and with the running buffer (20min). CE system was operated under normal polarity and constant voltage conditions of 30 kV. Samples were hydrodynamically injected into the capillary (50 mbar, 30 s). For the preparation of all solutions only deionized water was used. For pH adjustment a Crison Instruments BASIC 20 pH-meter was used.

2.2 Sample preparation

Analysis of a complex matrix (plant extract) requires precise step of sample preparation. The air-dried Parsley leaves (0.05 g) were powdered and extracted with methanol. The extraction was carried out for 30 minutes. Plant extracts were then hydrolysed with 12 M hydrochloric acid for 3 h in 85°C. Hydrates were, in the last step, centrifuged (12000 × *g*) and resulting supernatants were injected into electrophoretic system.

3 Results and Discussion

3.1 Effects of pH and buffer concentration

In order to improve the method efficiency, the effect of concentration and pH of the buffer solution were investigated. These parameters influence among other on EOF rate, the overall charge and thus migration time of the analyte. The effect of the buffer solution pH was evaluated in pH range from 9 to 11. A concentration of $Na_2B_4O_7 \cdot 10H_2O$ was also tested. The 30 mM borate buffer solution pH 10.2 was chosen as the best BGE concentration and pH in our experiments.

3.2 Effects of SDS and acetonitrile

Different SDS concentrations from the range 5-30 mM were used to study the effect of SDS concentration on separation. Both, migration time and area of apigenin signal increase with increasing concentrations of SDS. 10 mM SDS concentration proved to be the most appropriate to separate signals. Organic modifier also plays an important role in successful separation. 10% addition of ACN results in a good separation of apigenin peak from the matrix components.

3.3 Analysis performance

Standard addition method was used for calibration of the method. Peaks of analytes were identified by comparison of their migration times with those of standards. The LOQ, determined by spiking a proxy matrix (Ringer's solution) with decreasing concentrations of analyte, for apigenin was 1.0 μM. Calibration curve was constructed over the concentration range from 56 to 225 mg/g plant (y=5.3879x+570.95). The calibration plot was linear with correlation coefficient > 0.989. The recoveries for apigenin were between 86.2% and 104.8%.

4 Conclusions

We have developed simple and reproducible CE procedure for apigenin determination in plant extracts. Application of an appropriate mode of extraction allowed to use on-line stacking method for the determination of apigenin in *Petroselinum crispum.* Procedure allows approximately 20-fold increase of method sensitivity. The limits of detection and quantification were 0.5 μM and 1.0 μM, respectively. The results confirm that the method is of high separation efficiency, good repeatebility and convenient use. Elaborated assay can be useful for the determination of apigenin in biological samples.

Acknowledgement

This work was supported in part by grant No 545/1151 from the University of Lodz.

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P30 THE POSSIBILITIES OF FLUORESCENCE ANALYSIS OF SHORT NUCLEIC ACID BY ARTIFICIAL CHEMOSENSOR

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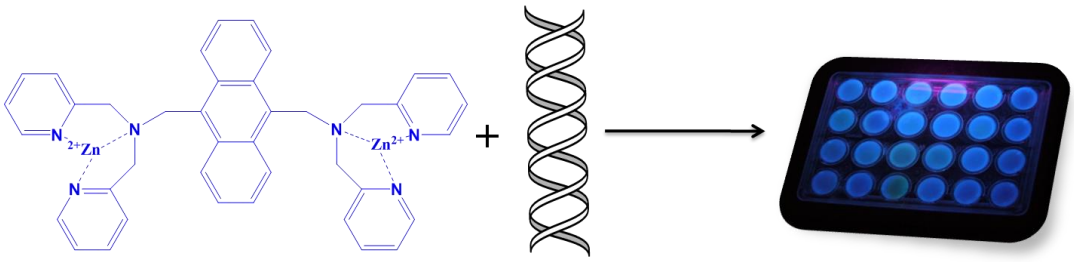
Summary



Chemosensor 1 **DNA** **Fluorescence analysis**

1 Introduction

The design and syntheses of receptors for the molecular recognition of anion is an important and difficult area of increasing research in supramolecular chemistry [1]. In recent years the field of research dealing with the molecular recognition of biologically important anions has been receiving increasing attention, considering the significant role anions play in nature: biologically, chemically, biomedically and environmentally.

The chemosensors in contrast to biosensors are synthetic sensors consisting biological structures, such as peptides, proteins and nucleic acids. Chemosensors are able to specifically bind to the target analyte and this change generates a measurable signal. In the design of chemosensors binding sites chemosensors must be considered, which must be able irreversibly and specifically bind to specific analytes. Several chemosensors have been designed, synthesized and have been used recently for anion recognition, for example for the visualization of ATP in living cells [2], the detection rate of phosphorylation of proteins [3], or to visualize the nucleic acids [4]. One of the recently described chemosensor is the fluorescent 9,10-bis[(2,2'-dipicolylamino)methyl] anthracene-zinc complex 1, prepared for detection of pyrophosphate anions in water. Chemosensor 1 selectively senses pyrophosphate anions and phosphorylated nucleosides with a large fluorescence enhancement [5-7]. We were interested if the chemosensor 1 is able to interact with short nucleic acid and can influence intensity of fluorescence.

2 Experimental

The fluorescent chemosensor 9,10-Bis[(2,2'-dipicolylamino)methyl]anthracene-zinc complex 1 was prepared in three steps starting from commercially available anthracene [6,8]. The complex 1 was used for the binding studies without further purification and the fluorescence spectra were recorded on Tecan Safire 2 Microplate Reader.

For measuring of fluorescence was used chemosensor 1 upon addition of anorganic pyrophosphates $(Na_2P_2O_7)^{2-}$, phosphorylated nucleosides (ADP, GTP, CTP, UTP, dATP, ATP) and model oligonucleotides:

oligo 8 5´ d(AGAGAAGGAAGGATTTTTCCTTCCTTCTCT) 3´
oligo 9 5´ d(TCT CTT CCT TC) 3´
oligo GA1 5´(GGA ATT CC)3´

3 Results and Discussion

The effect of short oligonucleotides on intensity of fluorescence of chemosensor 1 was measured in two ways. First, the ideal candidates for the simple initial fluorescence detection were phosphorylated nucleosides. The chemosensor 1 is known to exhibited an enhacement in fluorescence intensity upon addition of anorganic pyrophosphates. Thus, the fluorescence emission from chemosensor 1 was expected to increase upon addition of chosen nucleotides. Indeed, titration of chemosensor 1 with chosen nucleotides moderate to large fluorescence enhancements. Specifically, addition of equimolar concentration of adenosine triphosphate (ATP) to the solution of chemosensor 1 increased intensity of fluorescent by about 500% (Fig.1). Then, we were interested if the addition of short oligonucleotide influence fluorescent intensity. In this case, for the initial study on intensity of fluorescence of chemosensor 1 was used oligonucleotides with different lenght (11 and 30nt). The results from fluorescence spectra indicate, that intensity of fluorescence of chemosensor 1 is increasing by lenght of chosen oligonucleotides (Fig.2). Furthermore, the fluorescence intensity of chemosensor 1 is directly proportional to the concentration of model oligonucleotide GA1. This was proved by titration experiments by adding oligonucleotide GA1 in concentration range from 36nM to 216 nM to a 10nM solution of chemosensor 1 (Fig.3).

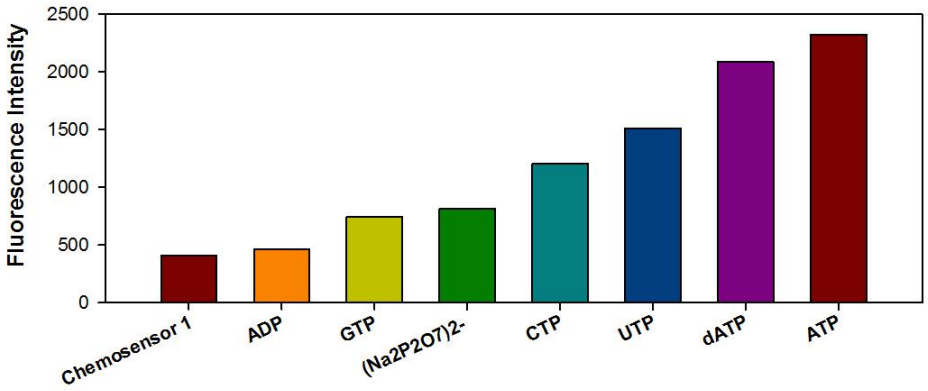


Fig. 1. Fluorescence intensity of 1 [1μM] upon addition of ADP, GTP, anorganic pyrophosphate, CTP, UTP,dATP, ATP; [add]= 1μM, (λex=380nm, λem=434nm).

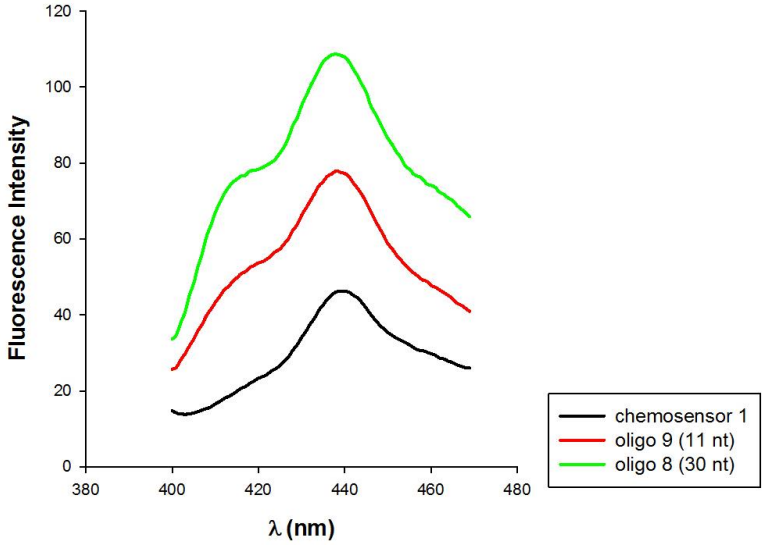


Fig. 2. Fluorescence spectral change of receptor 1 [0,5μM] upon the addition of oligo 8 (30nt) and oligo 9 (11nt); [oligo]= 200 ng/μl (λ_{ex}=380nm, λ_{em}=400-470nm).

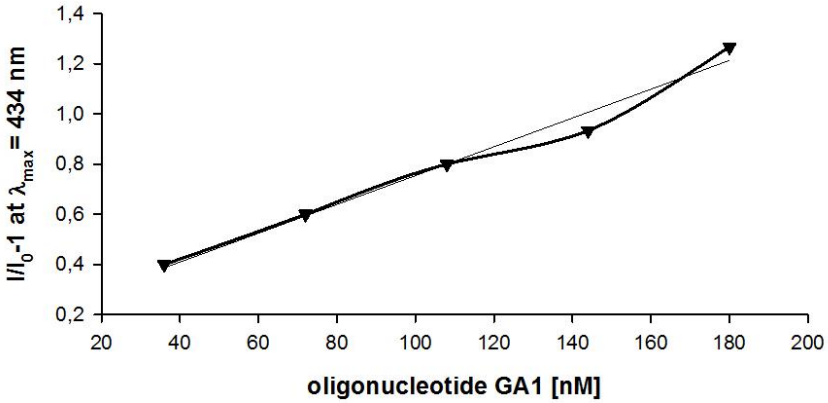


Fig. 3. Fluorescent titration curve of receptor 1 [10nM] upon the addition of oligonucleotide GA1; [GA1]: 36, 72, 108, 144, 180, 216 nM; in 5M urea (λ_{ex}=380nm, λ_{em}=434nm).

4 Conclusions

In conclusion, we have shown that the fluorescence intensity of chemosensor 1 is increased by phosphorylated nucleosides. Moreover, there is a significant fluorescence enhancement when the short nucleic acids are added to the solution of chemosensor 1. The magnitude of the enhancement depends on the length of oligonucleotide as well as on the concentration. This results indicate that chemosensor 1 is able to bind and recognise short nucleic acids, but we don´t know the way of binding of the chemosensor 1 in nucleic acid. Therefore, future studies will be focused on study of chemosensor -oligonucleotide complex structure.

Acknowledgement

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P31 THEORETICAL PRINCIPLES OF CAPILLARY ISOTACHOPHORESIS IN MOVING-BOUNDARY SYSTEMS

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Summary

This contribution provides fundamental theoretical background of ITP in moving-boundary systems. It is based on the approximation of capillary ITP as a 1D process

and the general condition of ITP migration, i.e., the existence of a self-sharpening boundary capable to form a stack of zones moving with equal velocities.

1 Introduction

Capillary isotachophoresis (ITP) is a well-established electrophoretic technique with high potential of on-line analyte enrichment [1]. Its sample stacking capabilities are nowadays employed predominantly by its combination with other separation techniques and/or various universal (e.g. contactless conductivity) or selective (e.g. mass spectrometry) detection principles [2]. This often requires advanced manipulation of selectivity and tuning of the system's stacking window. We have recently shown [3,4] that a promising solution is the application of a generalized concept of ITP performed in more complex electrolyte setups based on moving boundary systems.

2 Experimental

Calculations were performed with the freeware program Simul 5 Complex [5].

3 Results and Discussion

We use the very simple model of ITP in a moving boundary system with binary co-ion shown in Figure. 1.

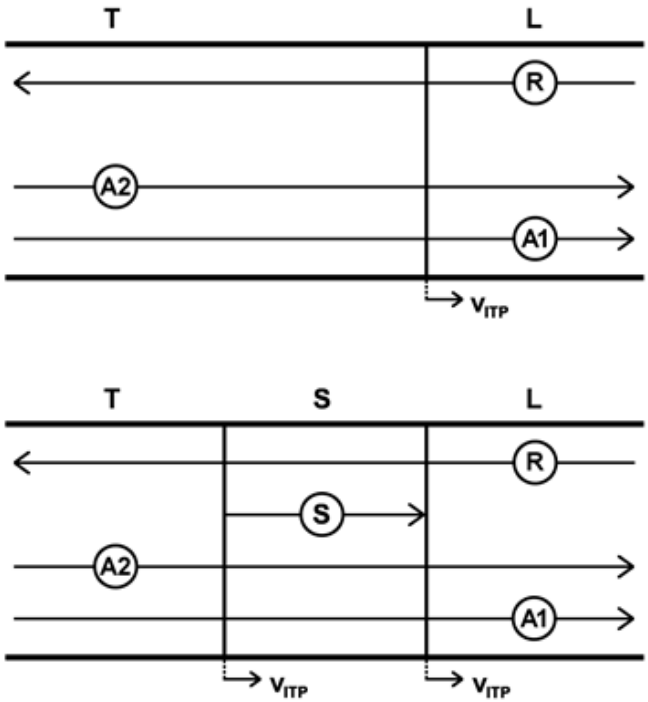

Fig. 1. Scheme of the system that includes two strong acids HA1, HA2 present in both leading (L) and terminating (T) zones (coions A1-, A2-), a strong base R (counterion RH+), and an analyte HS (strong acid, anion S-).

Using this example, the basic relationships describing the system properties can be derived from the appropriate set of moving-boundary equations. Such moving-boundary ITP (MB-ITP) system is defined by the composition of the leading zone and concentration ratios of A1 and A2 in the leading (L) and terminating (T) zones (a_L and a_T):

$$c_{A2,L} = a_L\, c_{A1,L} \qquad c_{A1,T} = a_T\, c_{A2,T} \tag{1}$$

The composition of zone T is then

$$c_{A2,T} = c_{A1,L}\,(u_{A1}a_2a_L + u_{A2}a_1)/(u_{A1}a_2 + u_{A2}a_1a_T) \tag{2}$$

where $a_1 = u_{A1} + u_{RH}$, $a_2 = u_{A2} + u_{RH}$, and u_i is the mobility of ion i. The condition of a sharp (self-sharpening) boundary can be written here as

$$u_{A2} < u_{A1} \qquad a_La_T < 1 \tag{3}$$

and the zone-related boundary mobilities [2,3] are then

$$u_{ITP,L} = (u_{A1}a_2 + u_{A2}a_1a_T) / (a_1a_T + a_2) \tag{4a}$$
$$u_{ITP,T} = (u_{A1}a_2a_L + u_{A2}a_1) / (a_1 + a_2a_L) \tag{4b}$$

where $u_{ITP,j} = v_s\kappa_j/i$ is the ITP boundary mobility related to zone j, v_s is the ITP boundary velocity, κ_j si the specific conductivity of zone j and i is current density (note that surprisingly $u_{ITP,L}$ and $u_{ITP,T}$ do not depend on a_L and a_T, respectively).
The condition for ITP stacking of an analyte HS (with anion S^-) between zones L and T (i.e., of formation of its own ITP zone) can be written as:

$$u_{ITP,T} < u_S < u_{ITP,L} \tag{5}$$

If HS is present in a sufficient amount, it forms a stationary ITP zone S separated from zones L and T (see Fig. 1). Zone S is a mixed zone by nature and contains besides S^- also the system anions $A1^-$ and $A2^-$. Its composition depends on the compositions of both zones L and T, namely on a_L and a_T:

$$c_{S,S} = c_{A1,L}\,\frac{u_S}{(u_S + u_{RH})u_{ITP,L}}\left[a_1\,\frac{u_{ITP,L} - u_S}{u_{A1} - u_S} + a_2a_L\,\frac{u_{ITP,L} - u_S}{u_{A2} - u_S}\right] \tag{6}$$

Eq. 6 indicates that the range of stacked analytes S can be selectively changed. A change in composition of zone L (i.e., change of a_L) changes the value of $u_{ITP,T}$, which represents the lower margin value of the stacking window. In analogy, a change in composition of zone T (i.e., change of a_T) changes the value of $u_{ITP,L}$, which represents the upper margin value of the stacking window.

4 Conclusions

It is demonstrated that moving-boundary ITP systems behave in the same way as regular ITP systems. This includes also the principle of ITP concentration adjustment

except the fact that additional parameters apply, namely the concentration ratio(s) of the multiple co-ions in both the leading and terminating zones. Equations derived for the simplified case of strong electrolytes allow insight into the nature of the system and reveal unexpected properties. The simplicity of the presented relationships employs the recently introduced [3,4] concept of zone related mobility of an ITP boundary, defining a pair of mobility values related to the pair of zones forming the given boundary.

Acknowledgement
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P32 DETERMINATION OF DIAGNOSTICALLY IMPORTANT AMINO ACIDS IN SAMPLES OF BIOLOGICAL ORIGIN BY RP-HPLC USING A PRE-COLUMN DERIVATIZATION

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1 Introduction

Amino acids (AA) are the building blocks of proteins, enzymes and hormones. In the brain they carry out basic functions and are also significant inhibitory and excitatory neurotransmitters. The best known neurotransmitters include aspartic acid (Asp), glutamic acid (Glu), γ-aminobutyric acid (GABA), glycine (Gly). Dysregulation of Glu and GABA is results in many neurological diseases such as multiple sclerosis [1].

In the literature, RP-HPLC is prevalent method used for the separation of amino acids in biological samples with pre- or post-column derivatization. The most common detection techniques used for the determination of AA using RP-HPLC

method in biological samples are spectrophotometric, fluorometric and electrochemical detection [2-4].

In the present work we focused on the development of RP-HPLC method for the determination of selected AA, potentially present in samples (CLS, blood plasma, etc.) taken from patients suffering from diseases of the nervous system, using pre-column derivatization with less frequently used reagent diethyl ethoxymethylenemalonate (DEEMM).

2 Experimental

2.1 Instrumentation

The chromatographic system LaChrom Merck - Hitachi (Merck, Darmstadt, Germany) equipped with the quaternary pump L-7100, autosampler L-7200, column oven L-7300 (with an accuracy of ± 0.5°C), diode-array detector L-7450, fluorescence detector (FLD) L-7480, solvent degasser L-7612, interface D-7000, and software HSM v. 4.1 was used.

2.2 Method

The chromatographic separation of AA samples was performed using an analytical column Chromolith Performance RP-18e (100×4.6 mm) with the guard column (Chromolith RP-18e 5-4.6 Guard Cartridge). The flow rate was maintained at 1.00 ml min^{-1}. Mobile phase consisted of a mixture of phosphate buffer solution (pH 3.6) and methanol. Column oven temperature was maintained at 40.0 °C ± 0.1°C. Injection volume 10 µl was injected by the autosampler. Wavelength range of DAD was set to 280 - 800 nm and monitored wavelength was set to 280 nm.

3 Results and Discussion

During the development of gradient RP-HPLC method, we used the knowledge acquired from the available literature [5] and from our previous experience with the determination of AA in commercial products with high concentrations of humic substances [6].

After optimization of separation conditions of gradient RP-HPLC method, we focused on the determination of the most abundant AA in biological samples at trace concentration levels. Figure 1 shows the chromatographic profiles of just DEEMM reagent at 280 and 254 nm, and mixture of 7 investigated AA at 280 nm and solution of 23 AA standards after derivatization using DEEMM at 280 nm. The proposed RP-HPLC method was applied to the analysis of three samples of blood serum (samples: 1, 2 - ill patient and 3 - healthy patient). All cases involved women between 25-35 years.

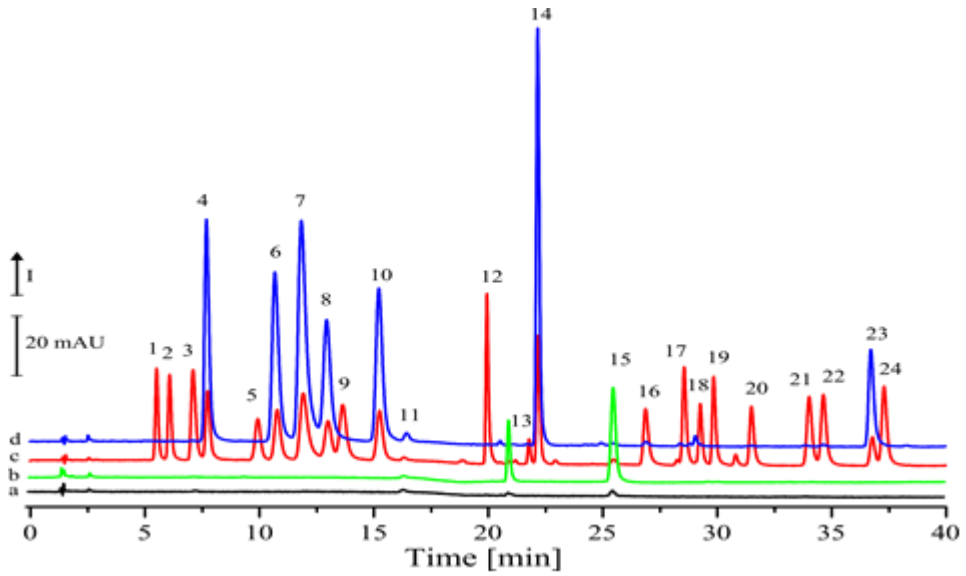

Fig. 1. Chromatographic profiles of DEEMM reagent recorded at 280 nm (a), 254 nm (b), mixture of solutions of 23 AA standards (c) and mixture of solutions of 7 studied AA standards (d) after derivatization of DEEMM at 280 nm. Elution order of AA: 1-histidine, 2-asparagine, 3-serine, 4-glutamine, 5-arginine, 6-aspartic acid, 7-glycine, 8-citrulline, 9-threonine, 10-glutamic acid, 11-NH_4OH, 12-alanine, 13-tyrosine, 14-GABA, 15–DEEMM, 16-methionine, 17-valine, 18-tryptophan, 19-cysteine, 20-phenylalanine, 21-isoleucine, 22-leucine, 23-homocystine, 24-lysine.

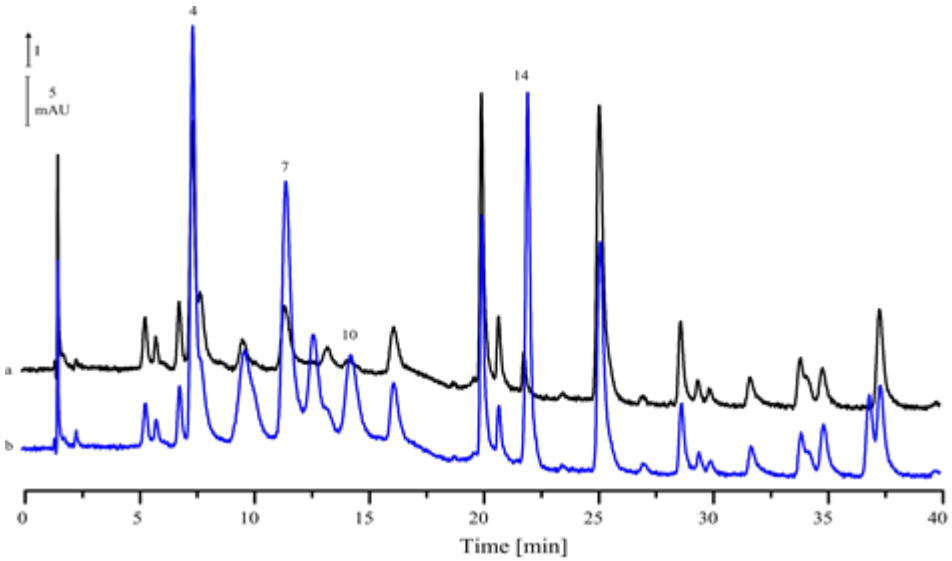

Fig. 2. Chromatographic profiles of blood sample 2 (a) and blood sample 2 with the addition of standards of seven studied AA (b) at 280 nm. Elution order of investigated AA: 4–gln, 7–gly, 10–glu, 14–GABA.

Table 2. Statistical evaluation of obtained results for diagnostically important AA in investigated blood samples calculated from 7 point calibration dependence.

Sample	Analyte	c [mg L^{-1}]	c [μmol L^{-1}]	R^2	Recovery [%] ± SD
1	Gln	2.4460	10.0418	0.9939	99.4 ±2.07
	Gly	0.7809	6.2407	0.9999	100.2 ±0.55
	Glu	0.4640	1.8922	1.0000	100.0 ±0.02
	GABA	0.2900	1.1004	0.9999	99.8 ±0.54
2	Gln	2.3449	9.6265	1.0000	100.0 ±0.1
	Gly	0.6553	5.2376	0.9995	100.4±1.08
	Glu	0.2852	1.1629	0.9985	100.9 ±2.39
	GABA	0.2784	1.6198	0.9998	99.7 ±0.93
3	Gln	1.7847	7.2727	0.9993	100.0 ±0.77
	Gly	1.0041	8.0254	0.9979	99.4 ±1.98
	Glu	0.2292	0.9346	1.0000	100.0 ±0.06
	GABA	0.2509	1.4598	1.0000	100.1 ±0.25

c – concentration; R^2 – correlation coefficient; SD – standard deviation.

When analyzing blood serum, we tried to analyze the content of 7 selected AA (see Fig. 1) not bounded to blood peptide or protein structures. Figure 2 shows a blood serum sample without the addition of the standards (a) and with the addition of the standards of seven studied AA (b). The comparison of the correlation of retention data and UV spectra with standards (DEEMM creates with compounds containing primary and secondary amino group stable products with a characteristic spectral band at 280 nm.) confirmed the presence of selected amino acids (Gln, Gly, Glu and GABA) in blood samples. The results were evaluated both, by the method of the calibration curve and the method of the standard addition. The calculated concentrations of selected neurotransmitters (AA) in blood serum samples are summarized in Table 1.

4 Conclusions

The novel developed gradient RP-HPLC method provides the possibility to separate 23 selected AA and was successfully applied to the determination of 4 investigated AA neurotransmitters in blood sample after pre-column derivatization with DEEMM. The LOD and LOQ (calculated from 7 point calibration dependence) were assigned at intervals of 0.05 to 0.1 mg L^{-1} (LOD) and 0.16 to 0.33 mg L^{-1} (LOQ). Using the method of the standard addition were quantitated selected AA in the blood serum samples at concentration levels of 0.33 to 3.59 mg L^{-1}. Most represented AA in the blood samples were Gln, Gly, Glu and GABA.

Acknowledgement

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P33 TREATMENT AND FUNCTIONALIZATION STUDY OF PDMS MICROFLUIDIC DEVICES DESIGNED FOR RARE CELL CAPTURE

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Summary

A comparative study of different PDMS microchip surface oxidation methods is described. The hydroxyl groups generated by the oxidation process were crucial for further functionalization of the PDMS surface, especially in microfluidic devices for rare cell capture. Contact angle and X-ray photoelectron spectroscopy (XPS) measurements demonstrated that oxygen plasma treatment generated sufficient amount of hydroxyl groups on the PDMS surface in contrast to chemical peroxide based oxidation which did not. The oxidized surface was functionalized by epithelial cell adhesion molecule (EpCAM) antibody and the microfluidic device was tested for cell capture with HT29 colon cancer cells. It was concluded that EpCAM antibody functionalization was only successful with oxygen plasma pretreated PDMS microfluidic device, but not with chemical peroxidase treatment.

1 Introduction

Poly-dimethylsiloxane (PDMS) is a widely used polymer for fabrication and especially rapid prototyping of microfluidic devices, because of its advantages of good elastomeric properties, biocompatibility, ease of molding into (sub)micrometer

features, simple bonding process to glass, relatively high chemical resistivity and low manufacturing cost [1]. However, the hydrophobicity of native PDMS is a significant disadvantage, so appropriate surface modification is required to improve surface wettability for facilitating liquid delivery and decreasing non-specific adsorption of hydrophobic analyte molecules. There are numerous surface modification techniques available to make PDMS surfaces hydrophilic [2], although the most widely reported methods to oxidize the surface are oxygen plasma treatment or chemical oxidation. This oxidation step is crucial for any further functionalization process, in our case to covalently bind antibodies to the PDMS surface [3, 4]. This work presents a comparison between oxygen plasma treatment and chemical oxidation techniques for consequent surface functionalization with EpCAM antibody for rare cell capture.

2 Experimental

For chemical oxidation, the PDMS sheets were immersed into H_2O:HCl:H_2O_2 (5:1:1) solution for 5 and 10 minutes. For oxygen plasma treatment, PDMS sheets were treated under 900 mTorr oxygen atmosphere for 1 and 2 minutes. Contact angle measurement and XPS spectroscopy techniques were used for the characterization of the oxidized PDMS surface. Contact angle measurements were carried out using the sessile drop method on a home-built goniometer (deionized water, 5 µl drop size). X-ray photoelectron spectroscopy (XPS) analysis was carried out on a KRATOS XSAM 800 XPS (Kratos Analytical Ltd, Manchaster, UK), using a monochromatized Al Kα source and each sample was scanned using a 20° incident angle. Signals were processed with the software provided by the manufacturer.

Functionalization of the oxygen treated PDMS surface with the antibody was performed within a PDMS microfluidic device [5]. Briefly, the microchannels were treated with 5% 3-aminopropyltriethoxysilane (APTES, Sigma-Aldrich, St Louis, MO) - ethanol solution for 30 minutes. The APTES-coated surface was then activated with 2% glutaraldehyde in water (AppliChem Lifescience, Darmstadt, Germany) for 2 hours. Next, the activated surface was incubated with recombinant protein G from Escherichia coli (Sigma-Aldrich) at a concentration of 50 µg ml^{-1} in PBS overnight at 4 ºC. The excess silanol sites were blocked with bovine serum albumin (BSA, Sigma-Aldrich) solution (2 mg ml^{-1} in PBS) and the system was incubated with the solution for 1 hour. EpCAM mouse antibody (Life Technologies, Carlsbad, CA) at a concentration of 100 µg ml^{-1} in PBS was then added to the protein G layer and incubated for 1 hour. After these functionalization steps the microchannels were washed with PBS [3]. The EpCAM antibody functionalized microchips were tested with HT29 colon cancer cell suspension (The HT29 colon cancer cell line was kindly provided by the National Institute of Oncology, Budapest, Hungary).

3 Results and Discussion

X-ray photoelectron spectroscopy measurement results revealed that the surface oxygen content of the plasma treated PDMS significantly increased, but the oxygen content of the chemically oxidized PDMS surface remained almost the same as the untreated one as shown in Table 1. In other words, chemical oxidation generated very

low density of hydroxyl groups, i.e., the silanization (with alkoxysilenes) and functionalization of the oxidized surface was less effective.

Table 1. Atomic percentages and carbon/oxygen (C/O) ratio of the PDMS surfaces from XPS scans.

Samples	C At%	O At%	C/O
Untreated PDSM	59.57	40.43	1.4734
PDMS treated with oxygen plasma 1 min.	57.28	42.72	1.3408
PDMS treated with oxygen plasma 2 min.	51.02	48.98	1.0416
PDMS treated with peroxide solution 5 min.	60.11	39.89	1.5069
PDMS treated with peroxide solution 10 min.	60.16	39.84	1.5100

The atomic percentage of the oxygen atom (O At%) increased with the time of the oxygen plasma treatment. On the other hand, in chemical peroxide solution oxidation the atomic percentage of the oxygen atom apparently did not change.

Contact angle measurements also confirmed that chemical oxidation of the PDMS surface was less effective. The contact angles after chemical oxidation neither decreased as much nor made the surface less hydrophilic (less hydroxyl groups) in comparison to the plasma treatment as shown in Table 2.

Table 2. Contact angle values of the PDMS samples.

Sample	Contact angle*
Untreated PDMS	97º
PDMS treated with oxygen plasma 1 min.	~10º
PDMS treated with oxygen plasma 2 min.	0º
PDMS treated with peroxide solution 5 min.	86º
PDMS treated with peroxide solution 10 min.	84º

* The measured contact angels were also dependent on surface roughness [6].

Although hydrophobic recovery was still an issue, after the oxidation treatment the functionalization had to be done immediately or the microfluidic channels must be filled with deionized water and stored at a constant low temperature to decrease hydrophobic recovery rate [7].

EpCAM antibody functionalization was accomplished for oxygen plasma and chemically treated microfluidic channels as well. After the antibody functionalization process, HT29 colon cancer cell suspension was pumped through the microfluidic channels at a flow rate of 20 μl h^{-1}. Cell capture abilities were recorded with a Nikon Eclipse TE200 Inverted Microscope (Nikon Corporation, Tokyo, Japan). It was observed that only the oxygen plasma pretreated microfluidic device worked adequately for cell capture, proving the successful functionalization of the EpCAM antibody (Figure 1).

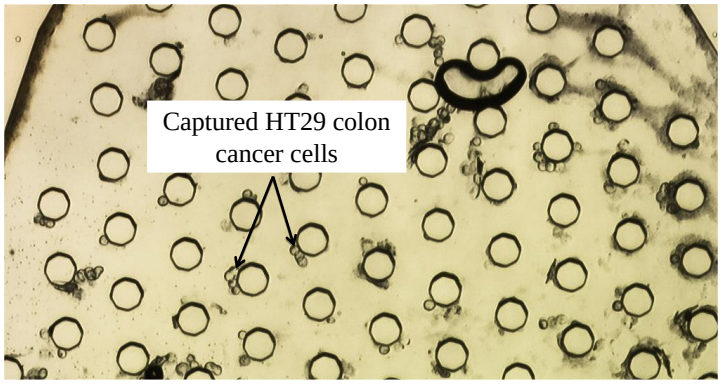

Fig. 1. Microscopic image of the successful cell capture by the oxygen plasma pretreated and EpCAM functionalized PDMS microfluidic.

4 Conclusions

Comparison of oxygen plasma and chemical oxidation techniques demonstrated that only the oxygen plasma treatment generated adequate number of hydroxyl groups on the PDMS surface for further antibody functionalization. The EpCAM functionalized PDMS based microfluidic devices proved to be useful for affinity based cell capture.

Acknowledgement

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P34 SEPARATION OF NONPOLAR LIPIDS FROM VERNIX CASEOSA

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Summary
The aim of this work is to develop a separation method for characterization of unknown nonpolar lipids in vernix caseosa. Here we optimized adsorption chromatography coupled to mass spectrometry detection with exact mass measurement. A long silica gel column (250 + 250 x 4.6 mm, particle size: 5μm) provided the best separation of nonpolar lipids present in vernix caseosa.

1 Introduction

Vernix caseosa is a multicomponent mixture, which is consisted of water (80 %) and proteins and lipids roughly in the same proportion (10 % each). This uniquely human material starts to be formed in the third trimester of pregnancy and is present on the skin of newborns after delivery [1, 2]. The lipids of vernix caseosa are classified as barrier lipids (cholesterol, free fatty acids, phospholipids, ceramides) and lipids originated from fetal sebaceous glands. Nonpolar lipids such as sterol esters, wax esters and triacylglycerols are dominant components of vernix caseosa [2, 3].

2 Experimental

2.1 Preparation of samples

Lipids from vernix caseosa were extracted by chloroform:methanol (2:1, v/v). A large-scale separation of lipids was carried out using classical low pressure column chromatography with 4.7 grams of lipids isolated from vernix caseosa. A silica gel column (length of glass column 41 cm, diam. 4.48 cm; particle size: 60-120 μm) with mobile phase hexane/diethyl ether gradient (from 1:99 to 50:50, v/v) was used to separate total lipid extract into fractions.

2.2 HPLC/APCI- MS/MS

The experiments were performed using LTQ Orbitrap XL hybrid FT mass spectrometer equipped with Ion Max source with APCI probe installed (Thermo Fisher Scientific, San Jose, CA, USA) and coupled to HPLC, which was consisted of a Rheos 2200 quaternary gradient pump (Flux Instruments, Reinach, Schwitzerland), PAL HTS autosampler (CTC Analytics, Zwingen, Schwitzerland); the system was controlled by Xcalibur software (Thermo Fisher Scientific). Samples were separated

using Acquity HILIC column (50 x 2.1 mm, particle size: 1.7 µm; Waters, Milford, MA, USA) or Spherisorb column (250 + 250 x 4.6 mm, particle size: 5 µm; Waters, Milford, MA, USA) at 30 °C. The gradient program for Acquity HILIC column, phase A (hexane), B (hexane/propan-2-ol, 96:4, v/v): 0 min: 99% A/1% B; 20 min: 85% A/15% B and 0 min: 99% A/1% B; 20 min: 66% A/34% B. The gradient program for Spherisorb column, phase A (hexane), B (hexane/propan-2-ol, 96:4, v/v): 0 min: 96% A/4% B; 60 min: 53% A/47% B. The mobile phase flow rate was 1.0 mL/min and the injected volume of samples was 10 µl in each chromatography. The APCI vaporiser and heated capillary temperatures were set to 270 °C and 170 °C, respectively. Nitrogen served both as the sheath and auxiliary gas at a flow rate of 15 and 17 arbitrary units, respectively. The MS spectra of the positively charged ions were recorded from 250 to 2000 *m/z.*

3 Results and Discussion

The total lipid extract was separated into 30 fractions. In this work we focused on fractions no. 12 and no. 13, i.e., those containing lipids of low or moderate polarities.

We compared two silica-based analytical columns for their ability to separate neutral lipids in vernix caseosa fractions. For each column the mobile phase gradient was optimized to provide optimum chromatographic resolution. The Acquity HILIC column allowed for short analysis time, but the column was not able to sufficiently separate lipid components of the sample (Fig. 1). Significantly better results were achieved with two Spherisorb columns connected in series (Fig. 2). The chromatographic peaks corresponded to various lipid classes. They were characterized using high resolution/accurate mass measurement mass spectrometry. General elemental formulas for lipid classes were obtained in this way. Fraction no. 12 was found to contain lipids with four oxygens, mostly diol diesters, and other lipid classes. This fraction also contained 2,3-oxosqualne (confirmed by GC/MS) and unknown lipids with five oxygens. In the fraction no. 13 we identified 2,3-oxosqualne, diol diesters and lipids with four and five oxygens too, but these lipids exhibited different retention times. Therefore, we hypothesize that these lipids have different structure than those in the fraction no. 12.

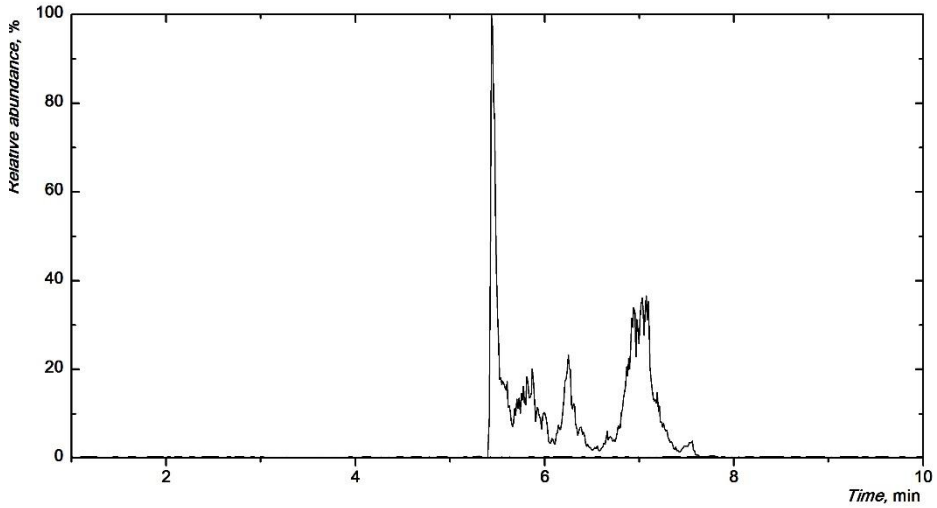

Fig. 1. Base peak chromatogram of the fraction no. 12 obtained using Acquity HILIC column (50 x 2.1 mm, particle size 1.7 µm).

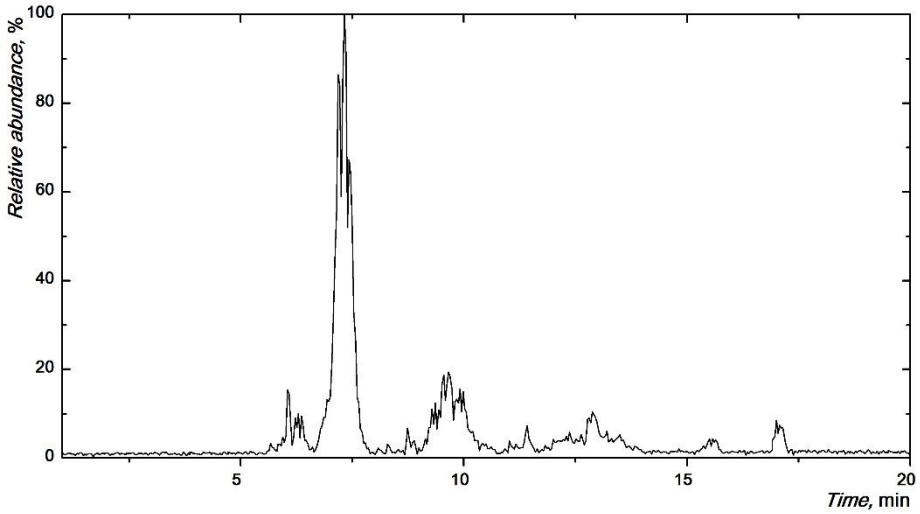

Fig. 2. Base peak chromatogram of the fraction no. 12 obtained using Spherisorb column (250 + 250 x 4.6 mm, particle size 5 µm).

4 Conclusions

Good separation of lipid classes of similar polarities and structures in complex lipid matrix of vernix caseosa was achieved using a 500 mm long silica gel column with a conventional diameter of 4.6 mm packed with 5 µm-particles. Attempts to use short column with small diameter particles were not successful. The lipids were characterized by their elemental formulas. The next experiments based on tandem mass spectrometry will be focused on disclosing their chemical structures.

Acknowledgement

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P35 PREPARATION OF LOW-COST MICROFLUIDIC DEVICES BASED ON FILTER AND BAKERY PAPERS BY USING AN OFFICE LAMINATOR

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Summary
In our work, we developed a new paper-based microfluidic device for determination of active content of common drugs. The device represents a fast, cheap and portable alternative for common analytical techniques used in forensic analysis. The main problem of injection into four parallel channels was overcame using a bakery paper integrated into the device.

1 Introduction

Miniaturization of analytical techniques is recently common and significant topic in analytical chemistry. The main aim is in enabling fast and cheap determination of water and food quality, medical diagnostics etc. without using instrumental devices. This could be very profitable not only for developed countries but also for the third world countries where the needs for low-cost analysis increase year by year. Martinez et al. [1] and Carillo et al. [2] described "paper-tape-based microfluidic devices" (μPADs), which can be easily produced for many purposes using filtration papers or nitrocellulose membranes.

In our work, we want to design microfluidic "paper-foil-based device" by using an office laminator, which will be able to determine active content of some mostly used drugs in medicine (for forensic screening purposes). Similar approach was presented recently by Cassano et al. [3] who called this device as "laminated paper-based analytical devices" (LPADs) and also by our group [4].

2 Experimental

Paper-foil microfluidic devices were prepared as follows: the final design was prepared as cut paper strips (as channels) and cut bakery paper strips, which were inserted into the laminating pouches Fellowes Impress A4 (100 µm) by tweezers and fixed inside by using the office laminator Fellowes Cosmic A4 (working at 120 °C). Standards and reagents used for the determination of active content of common drugs were as follows: acetaminophen, tramadol hydrochloride, cetirizine dihydrochloride, imipramine hydrochloride, acetylsalicylic acid (bought from Sigma-Aldrich, St. Louis, USA; all 1 $mg.mL^{-1}$), cyclohexane, ethyl acetate, and ammonia (bought from Sigma-Aldrich; all p.a. quality). A UV lamp working at 254 nm (P-LAB, Prague, Czech Republic) was used for detection of analytes.

3 Results and Discussion

First, we designed the device by using the office laminator. The laminator rollers worked at temperature of 120 °C. We found that the reagent zones were stable and laminating pouches protected ~~it~~ them from humidity, impurities and other pollution. The chip arrangement is in Fig. 1. The bakery paper ensured sample loading and allowed removal of dosing channel, which was used for injection of sample to those four separation channels by a single channel pipette from the left side. Then the separation channels were filled by the four different solvents using a multichannel pipette and the separation was performed.

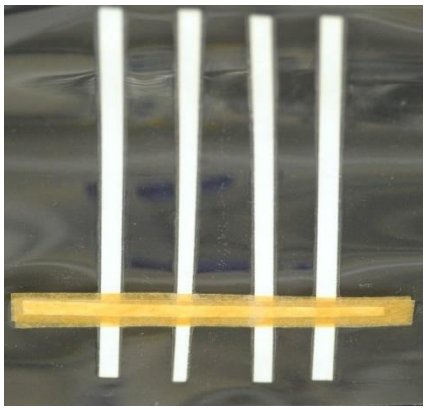

Fig.1. Microfluidic device for drugs analysis.

However, this arrangement was inappropriate for volatile solvents in the mobile phase. Analyses with volatile solvents in the mobile phase were performed on a chip illustrated in Fig. 2. In this approach, the sample was injected through the interlayer of filtration paper by a single channel pipette, which ensured injection of adequate volume of sample into separation channels.

Fig.2. Microfluidic device for volatile solvents.

The functionality verification of chip was performed on standards of acetaminophen, tramadol hydrochloride, cetirizine dihydrochloride, imipramine hydrochloride, acetylsalicylic acid all 1 $mg.mL^{-1}$. Universal mobile phase for basic determination of drug content consisting of cyclohexane, ethyl acetate, and ammonia (20:20:1) was injected in down part of the chip (to separation channels). Laminating pouch was cut to disclose of separation channels. The whole analysis was 30 s long, but the time of analysis depended on the length of separation channels. The UV lamp was used for detection of analytes.

4 Conclusions

We successfully developed a cheap paper-foil-based and easy to use microfluidic device for determination of main active content of common drugs. This chip can be easily used for toxicological screening of abused drugs.

Acknowledgement

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P36 VOLTAMETRIC DETECTION OF DNA DELETION ON PENCIL ELECTRODE

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Summary
In this work, we present a new qualitative approach of detection of PCR products using electrochemistry on pencil electrodes. PCR products with an incorporated biotin-labeled dNTP (dCTP in this study) are detected via conjugated streptavidine-alkaline phospatase. 1-Naphthyl phosphate (1-NP), which is dephosphorylated by alkaline phosphatase to release 1-naphthol, was used as a substrate in electrochemical detection of the PCR product. Voltammetric measurement on disposable pencil electrode is a very cheap and easy to use method. The system was optimized for plasmid DNA PCR product with potential to detect human genome DNA products and possible application in gene deletion monitoring.

1 Introduction

Enzyme linked assays are very sensitive due to amplification of signal and can be coupled to DNA hybridization using probe labeled with the enzyme via biotin-streptavidin interaction. Incorporation of biotinylated nucleotides in PCR products is possible with different efficiency depending on DNA polymerase, conditions of PCR or biotin-labeled nucleotide used [1]. The electrochemical measurement of biotin-streptavidin-alkaline phosphatase system was described earlier on screen printed carbon electrodes with polymerase extension reaction [2]. Here, the use of disposable pencil electrode and the incorporation in PCR product is studied.

2 Experimental

2.1 PCR with biotinylated dCTP

Amplification of the fr^{p53} was carried out in 50 µl reaction including: primers p53for and p53rev (0,25 µM each), *Pfu* DNA polymerase (0,3 U) , *Pfu* DNA polymerase buffer, dNTPs (100 µM of each with 2% biotinylated dCTP). pT77 template and pBluescript template was at final concentration of 0,01 ng/µl. PCR involved 30 cycles (denaturation 95 °C/90 s, annealing 71,4 °C 60s, polymerization 72°C/60s). Every PCR product was controlled by electrophoresis on agarose gel.

2.2 Electrochemical analysis

The amplified PCR product was cleaned by QIAquick PCR Purification Kit (QIAGEN), and the concentration of the cleaned product was measured on Nanodrop. Target DNA in 0.3 M NaCl solution was absorbed on pencil electrode for 5 min,

rinsed in 1x PBS and incubated in 5% streptavidin linked alkaline phosphatase solution in 5% milk for 1 min, rinsed again and measured in buffer 0.5 mM 1-NP, 0.5 M Na_2CO_3, 0.5 M $NaHCO_3$, pH9.5. The electroactive indicator 1-naphtol was detected after 60 s by linear sweap voltammetry with initial potential 0 V, end potential +0.9 V, scan rate $1V.s^{-1}$.

3 Results and Discussion

The oxidation of 1-naphtol at the pencil electrode produces a signal around 0.33 V. Every measurement needs the freshly prepared solution of buffer containing 0.5 mM 1-NP. The developed system uses small amount of enzyme, 1-NP and disposable pencil electrode what makes it very cheap and quick. The incorporation of biotinylated dCTP during PCR reaction with 2% of dCTP is sufficient for PCR product detection and resolution from negative control sample of pBluescript by optimized voltammetric measurement.

4 Conclusions

We present electrochemical enzyme-linked DNA sensing procedure based on application of disposable pencil electrode. This method enables the detection of biotinylated PCR product at a very small concentration and with high specificity.

Acknowledgement

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P37 ENOLISATION-SILYLATION REACTION STUDY OF SELECTED STEROIDS

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Summary
For the purposes of trace analysis of 1,4-androstadiene-3,17-dione (ADD), 1,4-androstadiene-3-one-17β-ol (Boldenone) and 17-β-estradiol in water, waste water, soil and for the further phytosterols transformation studies the enolisation-silylation reaction was studied using MSTFA and BSTFA with different catalysts as derivatization reagents. The different reaction conditions and stability of the products was also studied using GC-MS technique.

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^{-1}. PNEC value (Predicted No Effect Concentration) for 17β-estradiol (E2) is 1 ng L^{-1} (3.67 pM) [1].
Practical trace analysis is 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. For the derivatization of keto groups could be the enolization-silylation reaction the right derivatization step [2]. Enolisation-silylation is a complicated reaction by which the keto group of steroid hormones is converted into its enol tautomers. There is no information in the literature regarding the optimal conditions of this reaction, e.g. temperature of silylation, time of reaction and stability of the derivatives during their storage. Therefore, the main goals of our study were to a) test different reagents available on the market possibly suitable for this complicated enolisation-silylation reaction, b) find optimal conditions for the reaction and c) evaluate the stability of the final derivatives during storage. We have selected two compounds containing keto group for our study: the first having one keto group and one hydroxy group (Boldenone) and second one having two keto groups (ADD). 17β-estradiol was chosen for comparison of the hydroxyl group reactivity.

2 Experimental

Stock mixed solution of 17-β-estradiol, Boldenone and ADD at concentration 10 µg/mL in toluene was used for all experiments. Quantification was done using

internal standard calibration procedure using 5 calibration levels and cholesterol as internal standard. Prior to derivatization procedure, all glassware was silanized according to [3]. All experiments in this study were conducted in triplicates and samples were measured immediately after preparation.

For optimizing the derivatization process, the following reagents were tested for their ability to silylate the compounds of interest: MSTFA, MSTFA I (activated with ethanethiol and ammonium iodide), MSTFA II (activated with 2-(Trimethylsilyl)ethanethiol), MSTFA III (activated with imidazole), mixture of BSTFA and pyridine (1:1), MSTFA II and pyridine (1:1) (Fluka, Sigma-Aldrich). Derivatization was conducted as follows: 70 µL of sample was evaporated with a stream of nitrogen and afterwards redissolved with 50 µL of each reagent (and 50 µL of pyridine, when used). The mixtures were kept at 65°C for 60 minutes. Subsequently, they were evaporated and redissolved in 70 µL of hexane prior to GC-MS analyses. To optimize the derivatization procedure the set of duration and temperature combinations was tested using the MSTFA II derivatization procedure. The combinations of three temperatures - 55, 65 and 75°C, and three times - 30, 60 and 90 min, were tested. Finally, the in-time stability of the derivatives was examined using the MSTFA II derivatization procedure. The derivatives were stored dissolved in hexane at either -17° C or 4°C for 0, 1, 4, 15 or 40 days until analyses. The unique set of samples was prepared for each storage time.

The GC-MS measurements were conducted on ITQ 1100 mass spectrometer coupled with Trace GC Ultra gas chromatograph (ThermoFisher Scientific, MA, USA). The chromatographic separation was achieved on ZB-5 MS capillary column (Phenomenex®, CA, USA), 30 m × 0.25 mm× 0.25 µm. Helium was used as carrier gas at constant flow of 1 mL/min. The chromatographic conditions were set as follows: inlet temperature 275°C, splitless mode, transfer line temperature 250°C, temperature program - 130°C/0 min^{-1}, rate 25 °C min^{-1} to 235°C, then 2°C min^{-1} to 265°C, then 5°C min^{-1} to 290°C, then 10°C min^{-1} to 305°C holding 6 min.

3 Results and Discussion

The first part of this study focused on finding an optimum derivatization reagent for the three investigated compounds. The yield of 17-β-estradiol derivatization procedure was comparable among all tested reagents, with the exception of MSTFA III treatment, which yielded lower amounts of TMSi-derivatives ($F = 85.59$; $p<0.001$, Tukey HSD test). Nevertheless, the derivatization procedure was also reproducible (Fig. 1).

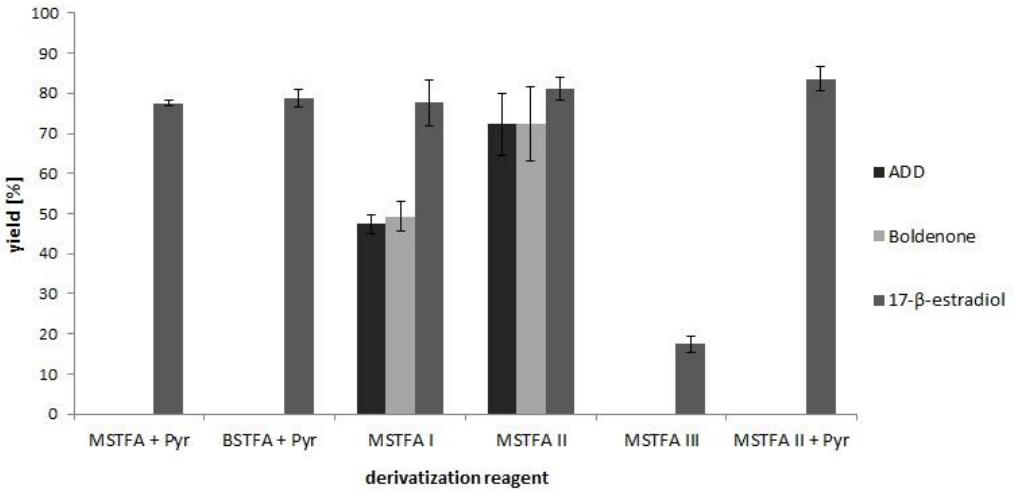

Fig. 1. Comparison of derivatization reagents used for enolisation-silylation.

Derivatization of steroid compounds containing keto groups was successfully achieved only when using MSTFA I or MSTFA II. However, the yields of ADD and Boldenone TMSi-derivatives were significantly higher when the derivatization protocol with MSTFA II was used (ADD: $F = 27.84$, $p<0.01$; Boldenone: $F = 16.1$, $p<0.05$, Tukey HSD test). The absence of derivatives when pyridine with MSTFA II was used (pyrdidine is recommended for routine silylation reactions) can be explained by the suppression of acidity of the hydrogen on the alpha carbon atom, and thus preventing the enolization reaction.

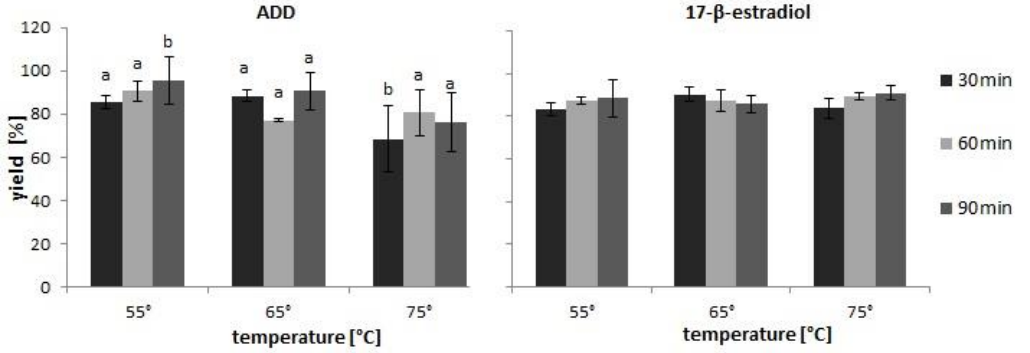

Fig. 2. Optimalization of time-temperature conditions during the derivatization process (only data for ADD and 17-β-estradiol are presented).

Subsequently, the optimum time-temperature combination for the derivatization process was examined. In the case of ADD and Boldenone a significant difference could only be observed between treatment at 55°C for 90 min and treatment at 75°C for 30 min with foremost treatment giving a significantly higher yield (ADD: $F = 2.69$, $p<0.05$; Boldenone: $F = 2.81$, $p<0.05$, Tukey HSD test) (Fig. 2). The range of tested time and temperature combinations during the derivatization process seems to have no effect on the yield of17-β-estradiolTMSi-derivatives.

Finally, the stability of TMSi-derivatives over time was studied. During the whole experiment, we didn´t find any significant difference between the two storage temperatures for none of these steroid compounds. However, a decrease of ADD and

Boldenone derivatives over time was detected. We determined similar degradation rate of these compounds which resulted in decay of approximately 35% of TMSi-derivatives over the 40 day experiment. Our results indicate stability of derivatized steroid compounds containing keto group for at least 4 days when stored in freezer while when stored in the fridge, the stability is maximum 1-2 days.

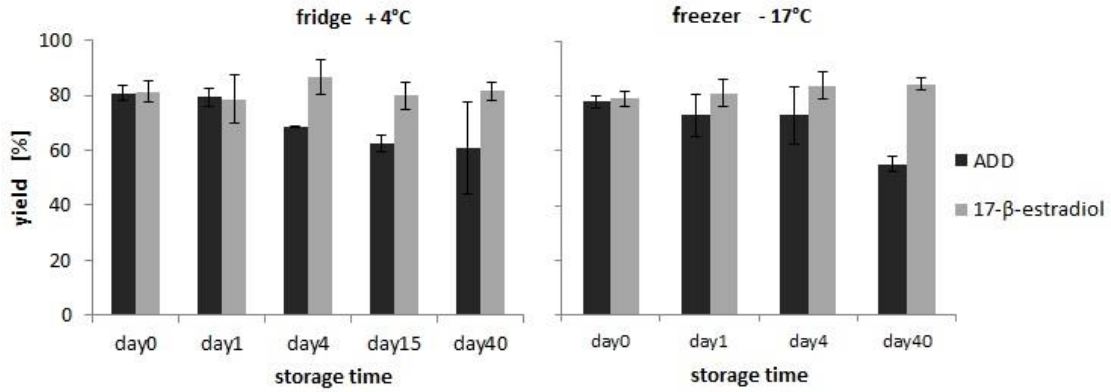

Fig. 3. Time stability of ADD and 17-β-estradiol TMSi-derivatives following storage at -17°C and 4°C.

4 Conclusions

Study of enolisation-silylation reaction revealed that it is possible to use this reaction for the determination of ADD and Boldenone and the reaction yield is comparable with estradiol using MSTFA activated II. Since the reduction of time needed for sample preparation is one of the main criterions during method optimization, the time-temperature combination of choice based on our results is at 65°C for 30 min. Due to instability of TMSi-derivatives, the immediate analysis after sample preparation is necessary.

Acknowledgement

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P38 PROTEIN EXTRACTION OF POPPY (*PAPAVER SOMNIFERUM*) FOR PROTEOMIC 2 – DE ANALYSIS

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1 Introduction

Strength of two-dimensional polyacrylamide gel electrophoresis (2D PAGE) is its ability to resolve and investigate the abundance of several thousand proteins in a single sample. Three different extraction procedures for two-dimensional electrophoresis of poppy are compared in this work. TCA/acetone-based and phenol-based extraction method, have been mainly used to extract proteins from different organs or tissues on many plant species. However, few results have been reported for poppy or another oilseed plants. We wanted to determine which of these protocols was optimal for oilseed plants in order to achieve both efficient protein extraction and high spot resolution on 2-D gels. The phenol-based protocol was superior to the TCA/acetone and sodium phosphatase methods, showing larger protein yields and greater spot resolution on 2-D gels.

2 Experimental

2.1 Sample

Seeds were used for extraction of proteins from seven Slovak registrated (Albín, Belgarn, Gerlach, Manor, Malsar, Maratón, Opál) and one Hungarian registrated (Buddha) varieties of poppy, which were obtained from GeneBank of Slovak Republic in Piešťany.

2.2 Extraction protocols

The first protocol used for extraction of the proteins was using phenol followed methanolic ammonium acetate precipitation [1– modified]. Plant tissue (100 mg) was homogenized well in the extraction buffer (0.1 M Tris – HCl pH 8.8, 10 mM EDTA, 0.4 % 2 – mercaptoethanol, 0.9 M sucrose) and the same volume of 0.4 M phenol buffer pH 8.8 was added. This mixture was shaken vigorously for 30 min at 4ºC and then centrifuged at 5000g for 10 min at 4ºC. The upper phenol phase containing the proteins was collected very carefully. Ammonium acetate (0.1 M) was added five times the volume of the phenol phase. Mixed well and kept for precipitation overnight at – 20ºC. Next day, the mixture was centrifuged at 5000 g for 20 min at 4ºC. The supernatant was discarded and precipitates were washed in 0.8 M acetone twice and once in 0.7 M ethanol.

The second protocol involved the extraction of albumin fraction of proteins using sodium phosphate. Homogenization of 100 mg of tissue was done in 25 mM sodium phosphate (pH 7.5) and mixed vigorously for 60 min at 4ºC. After that was centrifuged at 10000 g 10 min at 4ºC. As described for phenol extraction, the upper sodium phosphate phase containing the proteins was collected carefully and ammonium acetate (0.1 M) was added five times the volume of the phase. Mixed and kept for precipitation overnight at – 20ºC. Next day, the mixture was centrifuged 14000 g 20 min at 4ºC. The supernatant was discarded and precipitates were washed in 0.8 M acetone twice and once in 0.7 M ethanol.

The third TCA precipitation was based on the work of Görg *et al*. (2000). Briefly, 100 mg of homogenate tissue was precipitated overnight at – 20ºC with 13.3 % TCA in acetone containing 0.2 % DTT. Next day, the mixure was centrifugated at 14 000 g 20 min at 4ºC. The supernatant was discarded and precipitates were washed in 0.8 M acetone twice and once in 0.7 M ethanol.

2.3 Quantification

The proteins were quantified using Bradford reagent [3], samples were analyzed absorbance at 590 nm in triplicates.

2.4 Two – dimensional gel electrophoresis

The samples dissolved in lysis buffer were taken such that their concentration reached to 0.1 – 2.5 $mg.mL^{-1}$ for 2 – DE. This concentration of the sample was dissolved in rehydration buffer (8 M urea, 2 % CHAPS, 5 mM DTT, 0,2 % 3/10 ampholyte, 0,001 % Bromophenol blue). This buffer was stored in small aliquots as per requirement at -20ºC. The last two ingredients (DTT and ampholyte) were added fresh to the rehydration buffer just before use. A total of 315 μL of rehydration buffer containing the sample was evenly distributed in the rehydration strip holder. The ReadyStrip™ IPG Strip 17 cm (pH 3 – 10, Bio – Rad) was placed on it and this assembly was allowed to rehydrate passively overnight. Current of 50 mA strip as applied. The focusing conditions were: step 1 – 500V, step 2 – 1000V, step 3 – 4000V, step 4 – 8000V. The focused strips were first reduced in equilibration buffer (6 M urea, 50 mM Tris – HCl pH 8.8, 30 % glycerol and 2 % SDS) containing 50 mg DTT (added just prior to use) for 15 min on a gel rocker at room temperature. The reduced strips were then alkylated by adding fresh 1 g Iodoacetamide (IAA) at similar conditions. The reduced and alkylated strips were washed with 1x SDS buffer. These strips were then loaded onto 10 % SDS – PAGE without any stacking gel. This assembly was sealed using 1 % agarose sealing buffer. The gels were run, stained and destained just as for 1 – D electrophoresis. The gels were scanned using GS – 800™ Calibrated Imaging Densitometer (Bio – Rad).

3 Results and discussion

Plant tissues are rich in compounds that interfere with 2 – DE. These interfering compounds, e.g. polyphenols, terpenes, and organic acids, mainly accumulate in vacuole in various soluble forms, and are more abundant in green tissues than in

young seedlings or etiolated material [4]. Usually, two main strategies exist for removing these contaminants: removal before protein extraction or removal after protein extraction. Conventional removal of nonprotein contaminants involves the use of organic solvents (e.g. acetone, 10 % TCA in acetone) to wash contaminants out of tissue powder [5]. We extract proteins from dry power of poppy seedlings with a mixture of phenol, mixture of TCA/acetone and mixture of sodium phosphate. Phenol dissolves proteins (including membrane proteins) and lipids leaving water – soluble substances in aqueous phase, thus proteins in phenol phase are purified and concentrated together with cold methanolic ammonium acetate precipitation. Another advantage of phenol extraction is that it minimizes protein degradation often encountered during sample preparation, due to endogenous proteolytic activity [5]. Because of that, higher protein yields were achieved by phenol extraction protocol (Table 1).

Table 1. The protein content of the sample determined according to Bradford.

Variety	**c_pFeE [mg.ml^{-1}]^a**	**c_pFoE [mg.ml^{-1}]^b**	**c_p TCA [mg.ml^{-1}]^c**
Albín	2,54	1,33	1,39
Belgarn	1,87	1,36	1,62
Gerlach	1,74	1,53	1,37
Manor	1,55	1,09	1,31
Malsar	1,75	0,82	1,34
Maratón	1,53	1,52	1,43
Opal	1,65	0,82	1,14
Buddha	1,85	1,46	1,67
$x \pm \sigma^d$	1,80 ± 0,30	1,24 ± 0,28	1,40 ± 0,16
ν [%]^e	32,2	29,6	17,0

[a]protein content obtained by extraction with phenol, [b] protein content of the obtained phosphoric extraction, [c] protein content obtained TCA / acetone extraction, [d] arithmetic mean and standard deviation, [e] coefficient of variation

Based visualization of proteins for 2 – DE extracted sample is shown in Fig. 1 we evaluated phenolic extraction as the most efficient extraction of proteins from poppy seed.

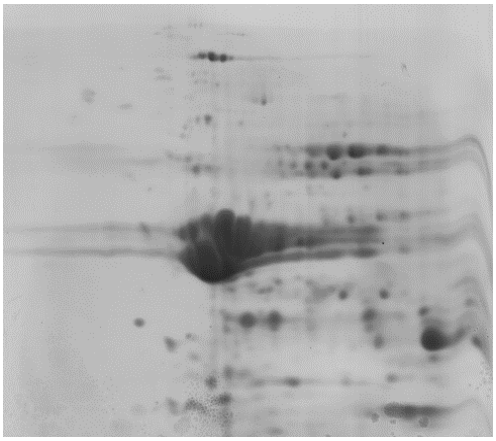

Fig. 1. Sample distribution proteome obtained by extraction with phenol (pI 3-10) 2 - DE (albin variety).

4 Conclusion

For qualitative identification of proteins in the seeds of poppy is the most important preparation and the amount of sample alone. We found out that the most appropriate protocol phenol extraction of proteins for the seeds of poppy in terms of quantity as well as quality visualization of total proteins for 2-DE analysis is according to Hurkmana and Tanaka.

Acknowledgments

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P39 SENSITIVE DETERMINATION OF GLUTATHIONE IN VARIOUS BIOLOGICAL SAMPLES BY CAPILLARY ELECTROPHORESIS WITH GREEN LASER INDUCED FLUORESCENCE DETECTION

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Summary
Laser induced fluorescence capillary electrophoresis (CE-LIF) with 515 nm green laser module was used for sensitive determination of glutathione (GSH) in various biological samples. For the first time, eosin-5-maleimide was used as a fluorescent tag in CE. Studies were performed to optimize the derivatization conditions (the ratio of reagent to analyte, the reaction time, temperature, etc.). CE-LIF also offers a unique opportunity to monitor the stability of the reagent and various reaction products. We demonstrate the applicability of the developed method on determination of GSH in real biological samples (exhaled breath condensate and saliva) with LODs as low as 180 pM.

1 Introduction

Glutathione (GSH) is an endogenous antioxidant that prevents the damage to cells from reactive oxygen species and free radicals. The decreased levels of GSH may indicate the presence of inflammation. GSH does not fluoresce in its native form and other physicochemical properties are not suitable for sensitive detection in the concentration range of GSH in various biological samples (nM) [1]. GSH can however be derivatized and detected by LIF. Eosin-5-maleimide (EMA) reacts selectively with thiol group on glutathione and glutathione can thus be detected by CE-LIF in various biological samples. Exhaled breath condensate (EBC) is an attractive, non-invasive, diagnostic sample obtained by condensation of exhaled breath. EBC contains small inorganic ions, larger organic molecules and peptides, proteins etc. The sampling of EBC is relatively simple procedure, in which a person breathes into a small sampling device described by Kubáň et al. [2].

2 Experimental

2.1 Electrophoretic system

The CE analysis was performed on home-built CE-LIF instrument shown in Fig.1 with an inexpensive 515 nm green laser module as a light source with exactly matched wavelength for EMA. A fused-silica separation capillary with 50 µm ID, 375 µm OD, 40 cm total length (Microquartz GmbH, Munich, Germany) was used. Hydrodynamic injection was used. Separation voltage was 15 kV. The optimized BGE consisted of 15 mM HEPES, adjusted with NaOH to pH 7.

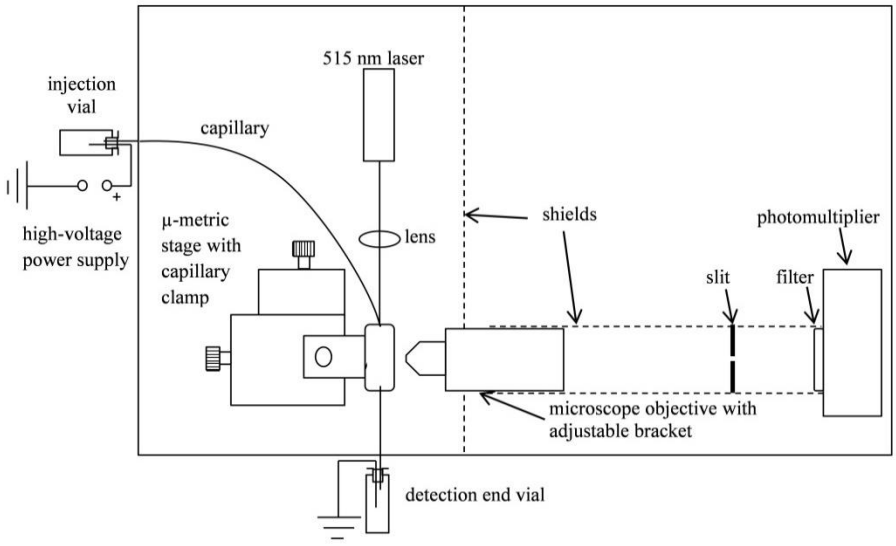

Fig. 1. Schematic diagram of CE-LIF instrument.

2.2 Sampling

The EBC sample was obtained from volunteers at Masaryk University by using a small sampling device [2]. Samples were derivatized immediately after the collection and stored in fridge at 4 °C. The saliva samples were centrifuged 2 min. at 7500 rpm, derivatized and stored at 4 °C.

3 Results and Discussion

3.1 Decomposition of EMA

EMA stock solution was dissolved in DMF and further diluted with water for the reaction with EBC, which is an aqueous solution. A problem with EMA stability in aqueous solution was observed as it was slowly converted to another form (EMA 2). The conversion in time is shown in series of electropherograms in Fig.2. The derivatization reaction must be therefore realized immediately after the dilution of EMA in water.

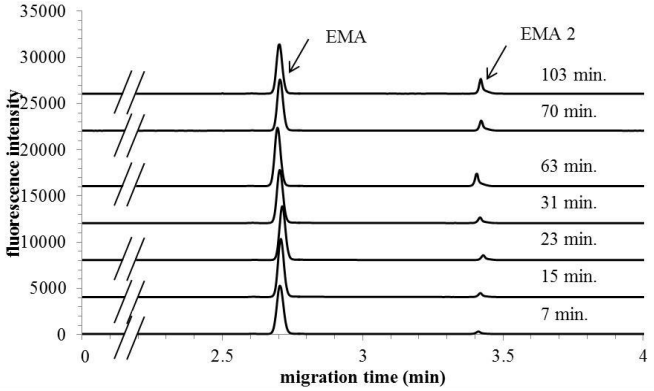

Fig. 2. Electropherograms of decomposition of EMA ($1 \cdot 10^{-7}$ M) at room temperature studied by CE. Separation conditions: 15 mM HEPES (pH=7), 15 kV, 35 s hydrostatic injection.

3.2 Optimization of derivatization time and pH

The optimum reaction time of EMA with GSH was studied. Reaction was performed at 4 °C and shielded from light by aluminium foil. The reaction was performed for 24 hours in all experiments. The influence of pH on the reaction gain was studied. The pH is important to provide an effective charge of EMA-GSH complex for the separation, as well as for the derivatization reaction to take place. Typically maleimide type reaction with thiol containing analytes takes place at pH close to 7. This is also beneficial for biological samples (EBC, saliva) and the reaction was perrofmed at this pH.

3.4 Analytical parameters of method

The analytical parameters of the developed CE method such as LOD, LOQ and repeatability (expressed as RSD of peak areas, n=10) are shown in Tab.1. The given repeatability was achieved with real samples of EBC containing the EMA-GSH complex. Limits of detection and quantification were sufficient to determine glutathione in real samples, such as EBC and diluted saliva.

Table 1. Analytical parameters of the developed method in real sample EBC.

LOD [nM]	LOQ [nM]	RSD	
		migration time	peak area
0.18	0.59	0.40%	2.76%

3.5 Analysis of glutathione in biological samples

The developed method was used to analyze various biological samples and we are for the first time showing the possibility to detect GSH in real EBC samples. The derivatized samples were analyzed by the developed CE method and the amount of GSH was quantified by using standard addition method. Fig.3 shows an example of the separation of EBC from a volunteer (lower trace) with subsequent standard additions of GSH to the sample. The obtained results demonstrate excellent sensitivity of the developed method. Tab. 2 provides quantitative analysis of GSH in EBC of five volunteers.

Table 2. Table of determined concentration of GSH in EBC of five volunteers.

	concentration of GSH in EBC [nM]			
volunteer 1	0.163	±	0.048	< LOD
volunteer 2	0.305	±	0.070	< LOQ
volunteer 3	1.61	±	0.82	> LOQ
volunteer 4	1.12	±	0.82	> LOQ
volunteer 5	1.69	±	0.44	> LOQ

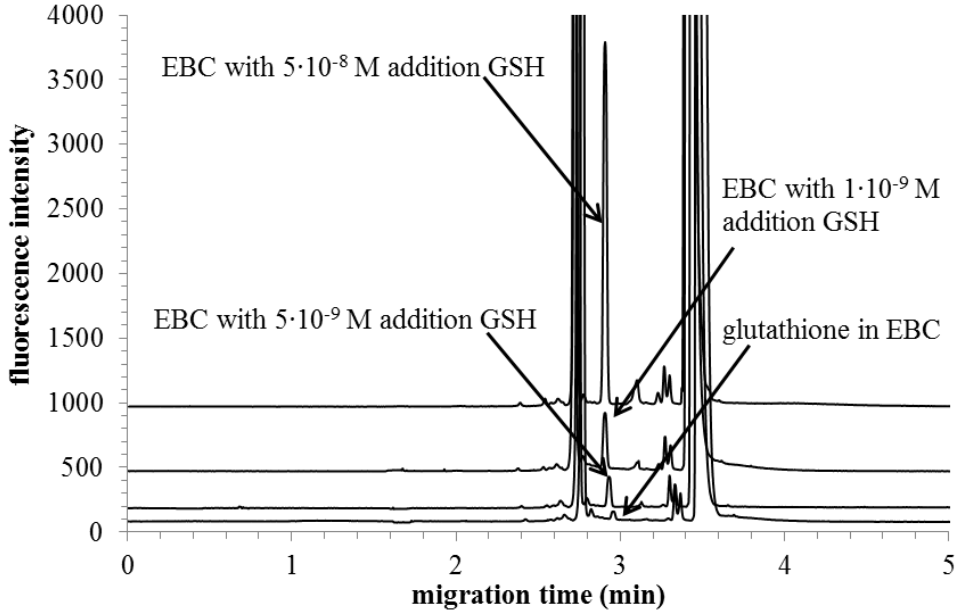

Fig. 3. Electropherograms of CE analysis of GSH in EBC with standard additions. Separation conditions: 15 mM HEPES (pH=7), 15 kV, 35 s hydrostatic injection.

The developed method can be used also for other types of samples. For instance, although the concentration of GSH in saliva samples is in the μM range, such samples cannot be directly injected into CE and must be diluted. By dilution the deteriorating effect of protein adsorption on the capillary wall can be eliminated. In Fig.4, we show a separation and determination of GSH in saliva sample diluted in ratio 25:1.

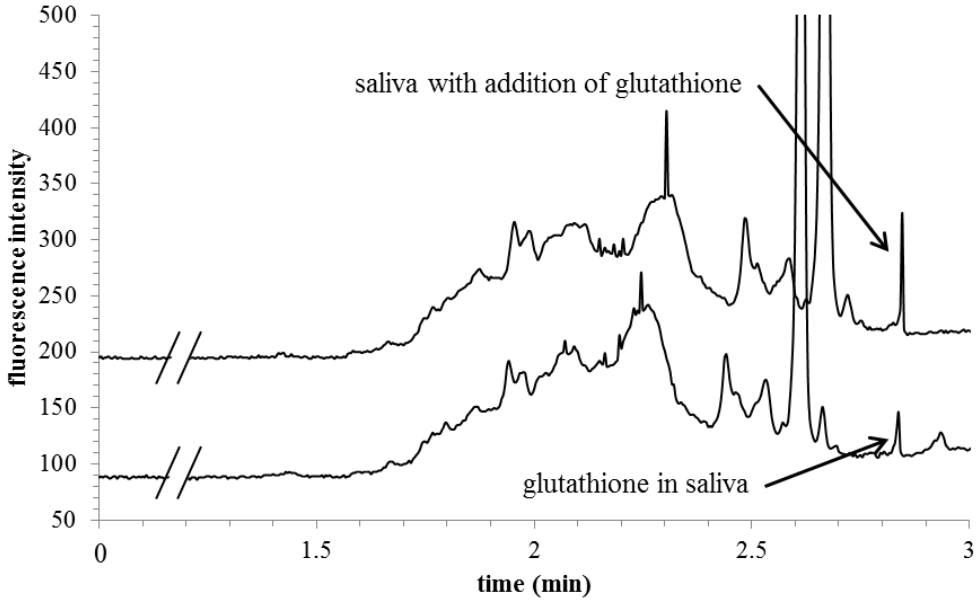

Fig. 4. Electropherograms of CE analysis of GSH in saliva with standard addition. Separation conditions: 15 mM HEPES (pH=7), 15 kV, 35 s hydrostatic injection.

4 Conclusion

In this work we have developed a new sensitive and repeatable method for quantitation of GSH in biological samples. For the first time it was possible to detect nM concentrations of GSH in EBC sample by CE-LIF. A new fluorescent reagent – EMA was used to successfully derivatize GSH. CE method with short separation time (4 min) and high sensitivity (LOD 180 pM) was developed. The method is applicable for other types of samples (saliva and possibly blood). A larger study in cooperation with Brno Faculty hospital is underway.

Acknowledgement

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P40 SUBCRITICAL EXTRACTION AS SAMPLE TREATMENT METHOD FOR ANTIOXIDANT SCREENING OF VARIOUS PLANT EXTRACTS

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Summary

Pressurized Hot Water Extraction performed in both static and dynamic modes followed by reversed phase HPLC with UV-visible detection was employed for fast and efficient determination of rutin and quercetin from the berries of *Aronia melanocarpa*, *Cornus mas*, *Hippophae rhamnoides* and *Sambucus nigra*. Moreover, the antioxidant power of subcritical extracts applying 2,2-diphenyl-1-picrylhydrazyl (˙DPPH)) was evaluated.

1 Introduction

The determination of high-value substances in plant materials has been of increasing interest in recent years because of their various interesting biological activities related to human health (e.g., reduction of coronary heart disease, anti-carcinogenic and

antioxidant properties). Therefore, rapid and efficient extraction procedure prior to chromatographic analysis of the sample is required [1]. Despite the dynamic progress of instrumental techniques the sample treatment is still an inseparable part of the analysis. The current trend to minimize the consumption of organic solvents with respect to environment, the lower time intensity and automation of the techniques play significant role also in this field. The liquid extraction methods at elevated temperature and pressure (Pressurized Hot Water Extraction, PHWE or Pressurized Fluid Extraction, PFE) largely meet these requirements and constitute an important part of analytical separation methodology [2, 3, 4]. The applicability of PHWE and PFE was summarized in recent reviews [5, 6, 7].

In this study, PHWE at static and dynamic arrangements was utilized for fast and efficient determination of rutin and quercetin flavonols from various plant materials. Moreover, the antioxidant activity of pressurized extracts applying 2,2-diphenyl-1-picrylhydrazyl ($^{\bullet}$DPPH)) was examined.

2 Experimental

2.1 Plant material

The berries of *Sambucus nigra, Cornus mas, Hippophae rhamnoides* and *Aronia melanocarpa* growing in the Czech Republic were collected during summer seasons 2008 and 2009, lyophilized and stored in brown glass vials at laboratory temperature.

2.2 Pressurized hot water extraction, PHWE

The PHWE was performed in static and dynamic arrangements. The static PHWE was performed as follows: A portion (2 g) of the sample was put into 11 mL extraction cell and extracted at a pressure of 15 MPa, temperature range from 40 °C to 120 °C with 20 °C steps, extraction time 5 min and number of cycles 1–3.

At dynamic arrangement, a portion (2 g) of the sample was put into 11 mL stainless steel extraction cell and extracted at various flow rates 0.2, 0.4 and 0.8 mL/min of pressurized water. The temperature ranged from 40 °C to 120 °C with 20 °C steps and the extracts were collected in the time range of 3–50 min.

2.3 HPLC-DAD analysis of PHWE extracts

The extracts were analyzed by HPLC-DAD system utilizing 3.0 mm i.d., 250 mm long Symmetry C18 5 µm stationary phase column at flow rate of 0.7 mL/min of gradient water/acetonitrile mixture adjusted at pH 2.3 by formic acid. The wavelength was set at 360 nm.

2.4 Determination of antiradical activity of PHWE extracts

1 mL of the extract diluted by water (1:499) was added to the same volume of 0.1 mM $^{\bullet}$DPPH solution. The mixture was shaken, left to stand in the dark at room temperature for 20 min and then absorbance was read at 517 nm. Antiradical activity was expressed as inhibition percentage (I %) of DPPH radical [8].

3 Results and Discussion

3.1 Optimization of static PHWE

The optimization experiments were performed with the berries of *Aronia melanocarpa*. The berries were extracted at 60 °C for 1×5, 2×5 and 3×5 minutes. The contents of all target analytes were increasing until the extraction period 3×5 minutes. The effect of temperature was studied in the temperature range 40–120 °C. The gradual increase of extraction yields of rutin and quercetin with temperature within the whole profile was noticed. The highest recovery of the target analytes was obtained at 120 °C.

3.2 PHWE at dynamic arrangement

The berries of *Aronia melanocarpa* were extracted by pressurized water at various flow rates 0.2, 0.4, and 0.8 mL/min. Based on previous experiments, the temperature was set to 120 °C. The increasing recovery of both rutin and quercetin with higher flow rate was observed, the transfer of both rutin and quercetin from the berries to pressurized water was controlled by solubility of the analytes in the solvent.

3.3 HPLC-DAD of plant materials

The berries of *Aronia melanocarpa*, *Sambucus nigra*, *Cornus mas* and *Hippophae rhamnoides* were analyzed by HPLC method after previous isolation of the target analytes by static PHWE under optimized conditions. The major substance of all samples was rutin, considerable amount of it was observed in the berries of *Sambucus nigra*.

3.4 Antiradical activity of PHWE plant extracts

The berries of *Aronia melanocarpa*, *Sambucus nigra*, *Cornus mas* and *Hippophae rhamnoides* were extracted by static PHWE at optimal conditions (temperature 120 °C, extraction time 3×5minutes). As reported in Fig. 1, antiradical activity of the extracts was expressed as inhibition percentage (*I* %) and ranged from 20 % (*Hippophae rhamnoides*) to 72 % (*Aronia melanocarpa*).

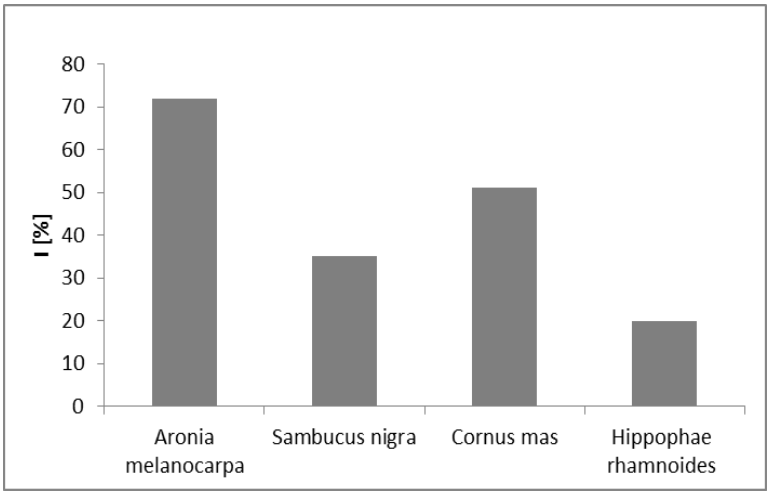

Fig. 1. Antiradical activity of the samples evaluated as inhibition percentage (*I* %) of DPPH radical.

4 Conclusions

It was shown that Pressurized Hot Water Extraction represents fast and effective sample treatment method for determination of biological active character of plant extracts. Moreover, the application of water in subcritical conditions at dynamic approach can provide valuable information about the extraction process.

Acknowledgment

The financial support of this work by the Czech Science Foundation (Project No. P106/12/0522), Academy of Sciences of the Czech Republic (Institutional Support RVO:68081715) and European Social Fund (Project No.CZ.1.07/2.3.00/20.0182 administered by the Ministry of Education, Youth and Sports of the Czech Republic) is gratefully acknowledged.

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P41 THE SELENIUM DETERMINATION IN LLAMA HAIR BY HYDRIDE GENERATION AAS

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Summary
The aim of the work was to develop a method for the assessment of selenium in alpaca hair. We optimized a cleaning procedure of the hair and sample processing (mineralization, evaporation, reduction) before measurement. Selenium was measured by Hydride Generation Atomic Absorption Spectroscopy technique (HG-AAS). We have found an average concentration of selenium in white hair 1289.13 ± 111.33 µg/kg and in brown hair 661.71 ± 43.56 µg/kg. The relative standard deviations (RSD) were 8.63% for white hair and 6.58% for brown hair.

1 Introduction

In recent years, the popularity of llamas is growing and the animals are kept in many European countries. A similar trend is in the Czech Republic as well, where llamas are keeping as a companion animals or for wool production. The most common health problems of llamas are based on the incorrect composition of their feeding; for example the trace elements deficiency that negatively affects animal health, reproductive function, and quality of produced wool.

Selenium is a trace element which deficiency occurs very often [1]. The evaluation of trace elements of interest is often determined in blood or organs. The blood sampling of llamas is relatively difficult and the need of animal fixation is very stressful for sensitive llamas. An alternative method for assessing the content of minerals in the body is the determination of minerals in the hair of animals. The hair taking is a non-invasive method without any pain and violent fixation. In this work the method of hair samples processing and selenium concentration determination by Hydride Generation AAS is presented.

2 Experimental

2.1 Sample processing

Cleaning procedure of hair samples is shown in the Table 1.

Washing solution	Procedure	Repetitions
0.5% Triton X-100	Washing 90 min.	3
0.5% Triton X-100	Washing 15 – 20 hours	1
0.5% Triton X-100	Washing 60 min.	2
Distilled water	Washing 60 min.	1
Acetone	Washing 60 min.	2
Distilled water	Washing 60 min.	1
	Drying (75°C) 60 – 90 min.	

2.2 Mineralization, evaporation and reduction of the samples

Mineralization of hair samples was performed by microwave digestion in Microwave Labstation Ethos SEL (Milestone, Italy). We used 0.5 g of treated hair together with 6 ml HNO_3 and 2 ml H_2O_2. The content was mineralized at 200°C for 35 minutes applying maximal power of 1000 W. The microwave programme was started by increasing the temperature over 20 min, followed by holding the temperature for additional 15 min. The resulting solutions were evaporated to clean samples from residues of nitric acid in the same equipment during approximately 25 minutes until a small droplet remains at the vessel.

Then the samples were diluted with deionised water into 25 ml volumetric flask. The reduction of Se^{6+} to Se^{4+} was performed by the addition of 12.5 ml 6M HCl for 20-24 hours in room temperature in the dark [2]. The reduced sample is stable up to three days at 8°C. The reaction is shown in the following equation:

$$HSeO_4^- + 3\ H^+ + 2\ Cl^- \rightarrow H_2SeO_3 + Cl_2 + H_2O$$

2.3 Measurement Se concentration by HG-AAS

Selenium concentration in reduced samples was measured by hydride generation atomic absorption spectrophotometry. The reaction is shown in the following equation:

$$H_2SeO_3 + 8\ H \rightarrow SeH_4 + 3\ H_2O$$

We used an Atomic Absorption Spectrometer ContrAA 700 (Analytik Jena, Germany) with hydride generation unit HS 600. This device has a unique lamp that illuminates a wide spectrum and it can also select very narrow part of the spectrum. Three pixels were chosen for selenium signal detection that is adequate to a slit width 0.0033 nm. All samples were measured in triplicates and the obtained values were processed by ASpect CS software, version 2.1.

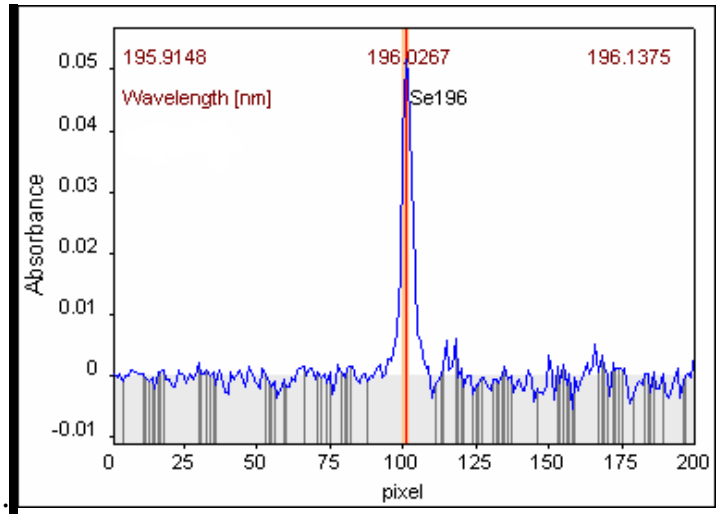

Fig. 1. Selenium signal determined by AAS - 3 pixels were chosen for detection (0.0033 nm).

3 Results and Discussion

The evaluation of the method was performed with two sets of alpaca hair samples from two different animals – white and brown alpaca. The whole procedure from mineralization to measurement was performed 10 times with both sample types. The concentration of selenium for white and brown hair was following: 1289.13 ± 111.33 µg/kg and 661.71 ± 43.56 µg/kg respectively. The relative standard deviations (RSD) were 8.63% for white hair and 6.58% for brown hair. Since the biological variability of animal hair is wide, the RSD lower than 10 % is acceptable.

Method validation was made by the determination of the detection limit, linearity of calibration, repeatability and recovery of the samples. Detection limit calculated from the calibration curve by the software was 1.40 µg/l. Since the level of Se concentration in alpaca hair is relatively high, this detection limit was sufficient. The calibration curve was linear up to 50 µg/l. The repeatability was determined by the triplicate measurement of every sample by hydride generation. The average RSD was 3.95% for white and 6.84% for brown hair.

The recovery was performed by addition of standard solution into the sample prior the mineralization. We found out the value of 109% that is acceptable mainly in connection with biological variability.

4 Conclusions

The presented developed method will be used to verify the diagnosis potential of the hair for the evaluation of the selenium intake by alpacas.

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P42 A PILOT STUDY ON DETERMINATION OF CITRATE AS THE STABILIZATION AGENT OF NANOPARTICLES USING CAPILLARY ZONE ELECTROPHORESIS WITH INDIRECT UV DETECTION

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Summary
Indirect UV detection in a co-electroosmotic mode in capillary zone electrophoresis (CZE) is frequently used for determination of small inorganic and organic anions in variety types of samples. Suitable separation factors, including a type of UV probe, relative mobility of the probe and the analyte and the type of electroosmotic flow modifier are discussed in many studies. In this work, we developed a simple and fast CZE method for determination of citrate in solution of nanoparticles. The CZE analysis was achieved using optimized background electrolyte containing 5 mM phthalic acid as the UV probe titrated by sodium hydroxide to pH 6.5. The electroosmotic flow (EOF) was reversed using cationic modifier polybrene (PB). The detection was at 200 nm.

1 Introduction

In recent years, capillary electrophoresis (CE) represents a valuable alternative to chromatographic techniques for the determination of small inorganic and organic anions, because this method provides fast analysis, large peak capacity, efficiencies of 10^6 theoretical plates and low consumption of chemicals [1]. The determination of these anions is frequently in a co-electroosmotic mode with indirect UV detection [2].

In the indirect UV detection, a background electrolyte (BGE) must contain UV-absorbing species (referred as the "UV probe" co-ion) to provide a background absorbance. In this case, detection is accomplished as a result of displacement of this UV probe ions by the analyte ions. For optimum peak shapes and highest sensitivity, the mobility of the analytes and the UV probe should be similar. Naturally, aromatic carboxylates such as phthalate or benzoate, chromate were employed as the UV probes [3, 4]. Also compounds affects (reverse) the electroosmotic flow must be added to the BGE. Generally, long chain alkyltrimethylammonium salts as cetyltrimethylammonium bromide (CTAB) [5] or cationic polymers as polybrene (PB) are used in order to reverse the eletroosmotic flow (EOF).

In this work, we describe the use of CZE method with direct UV detection for determination of citrate in a solution of nanoparticles. In-house synthesized silver nanoparticles stabilized during the synthesis by citric acid [6] and commercialized gold nanoparticles were analyzed. After washing steps, citrate amounts were directly determined by CZE.

2 Experimental

2.1 Chemicals and reagents

Electrolyte components: phthalate acid, sodium hydroxide, cetyltrimethylammonium bromide, dodecyldimethylammonium bromide and polybrene were purchased from Sigma Aldrich (St. Louis, MO, USA). The analytes standards: sodium chloride, sodium nitrate, ammonium molybdate, and citric acid were bought also from Sigma Aldrich. Samples: nanoparticles were synthetized according to the setup published by Panáček et al. [6] using glucose as a reduction agent. Gold nanoparticles were purchased from Sigma Aldrich. Stock standard solutions of all analytes were prepared at a concentration of 1 mg/mL in deionized water water (18 MΩ cm, 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 detection wavelength was set at 200 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 – 10 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), polybrene (30 min) and then with the running buffer (10 min). Polybrene was used for the coating of the capillary inner wall. 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 of the measurements were performed three times unless stated otherwise.

3 Results and Discussion

First, electrolyte systems for indirect UV detection were studied. Phthalate was chosen as the UV absorbing probe since it had the closest mobility to the analytes (citrate). In our next experiments, we tested various background electrolytes for the purpose of search for the optimal conditions. First, the effect of concentration of probe co-ion (1–30 mM) was studied. The best separation of all the analytes was observed for 5 mM concentration. High concentrations did not provide any separation. Then, the effect of EOF modifier was studied. We chose three EOF modifiers: CTAB and DDAB, which were presented directly in the electrolyte, and PB (Polybrene), which was used for coating of the capillary inner wall prior to the analysis. The best results were achieved with PB. Finally, the effect of pH was evaluated with sodium as counter-ions. The pH of 4.5; 5.0; 5.5; 6.0 and 6.5 was tested using NaOH solution for adjustment of the pH. The best separation according to the peaks' resolution and shape was observed at the pH 6.5. Therefore, the CZE was

performed using 5 mM phthalate/Na at pH 6.5 with following migration order: chloride, nitrate, molybdate and citrate (Fig. 1).

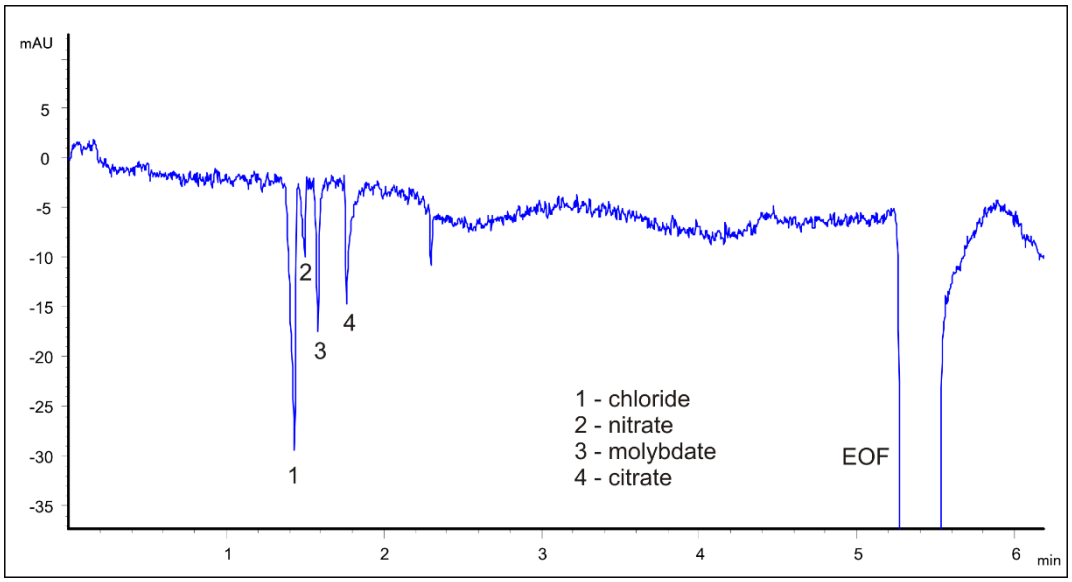

Fig. 1. Electropherogram of analyzed ions. Operating conditions: 5 mM phthalate buffer, pH 6.5, -10 kV, 25 °C, 5 s injection time, anions concentrations: 0.05 mg/mL

Under the best conditions, nanoparticles were analyzed. Two types of samples were studied: (a) nanoparticles stabilized by citrate and (b) those particles with addition of other buffers (based on acetic acid, boric acid etc.). Here, we observed significant changes in citrate amount that is being analyzed now.

4 Conclusions

In our study, an easy and fast CZE method for determination of citrate in solution of nanoparticles was developed and performed. We believe that this method can be used also for other studies, e.g. for characterization of the surface chemistry of nanoparticles.

Acknowledgement

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P43 IN-SYRINGE ANALYSIS / LAB-IN-A-SYRINGE: A NOVEL FLOW TECHNIQUE FOR LARGE VOLUME SAMPLE PRETREATMENT

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Summary
Complex samples and analytical tasks generally require a lasting sample pretreatment such as for digestion, dissolution, and matrix removal to make the analysis feasible as well as for analyte enrichment and derivatization to achieve reliable and sensitive analytical response.

In this poster, we overview the potentials and selected applications and operation modes of in-syringe analysis – also denoted lab-in-a-syringe technique – as a novel flow technique designed for the treatment of milliliter sample volumes and discuss its applicability and further potentials for sample pre-treatment.

1 Introduction

Sequential Injection Analysis (SIA) is an analytical flow technique used to automate sample pretreatment procedures such as solid phase extraction (SPE) and to achieve coupling of sample pre-treatment to chromatographic or electrophoretic separation [1, 2]. An automated syringe pump and a multiposition selection valve is used to accomplish sample and reagent handling in a tubing system including metering, mixing, dilution, analyte enrichment, derivatization, and measurement. General benefits of such automation are higher reproducibility, less solution consumption, and less risk of sample contamination. However, there is a limitation for the automation of procedures, in which homogenous mixing and use of large sample volumes is beneficial such as liquid-liquid micro-extraction (LLME) techniques.

In 2012, Maya et al. [3] demonstrated the potential benefits of using the syringe of an SIA system itself as extraction chamber for the automation of dispersive LLME. Further improvements included in-syringe detection [4] and using a magnetic stirring bar inside the syringe to achieve a size-adaptable reaction and mixing chamber [5].

The In-Syringe Analysis (ISA) technique (also Lab-In-Syringe) is overviewed in terms of construction and possible operation modes on the example of three reported applications including fully automated stirring-assisted LLME of aluminium

as lumogallion complex [5], LLME of cationic detergents including extract washing [6], and head-space single-drop extraction of ethanol from beverages [7].

2 Experimental

The three discussed analyzer configurations are given in Figure 1. An automated syringe pump and an rotary 8-port multiposition selection valve was used throughout. Inside the syringe of the syringe pump, a magnetic stirrer was placed and rotation was induced by the creation of an external rotating magnetic field.

All instrumentation was computer-controlled. Sample, reagents, and air were aspirated in the order as required from the multiposition valve into the syringe. A head-valve on the syringe further allowed emptying the syringe towards the detection flow cell and discharge the syringe content to waste.

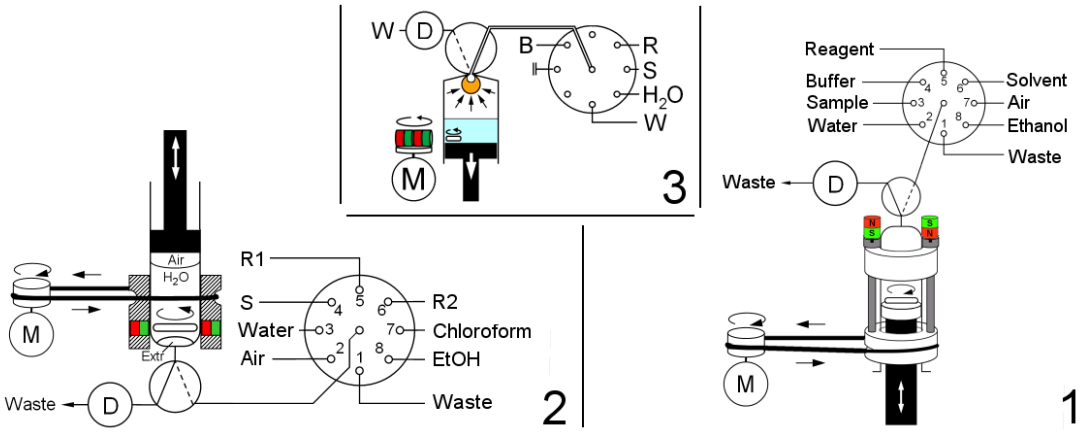

Fig 1. Different configurations for in-syringe analysis with magnetic stirring. 1: for solvents lighter than H_2O. 2: for solvent denser than H_2O. 3: Head-space extraction.

In the original configuration (Fig.1-1), a rotating magnetic field along the entire length of the syringe was created by placing onto the syringe barrel a device made of two plastic rings and two 8 cm long steel screws, magnetized with two neodymium magnets. The device was driven by a DC motor supplied via a computer controlled relay board. By this configuration, the stirring bar was forced to spin independently from the position of the syringe barrel. The configuration allowed mixing of sample and reagents and stirring assisted LLME. The configuration was used for the extraction of the complex of aluminium and lumogallion into n-hexanol as a solvent lighter than water with fluorescence quantification [5].

In a second development, the syringe was used upside down so that the stirring bar remained in the bottom position. This allowed to use a simpler device for the creation of the rotating magnetic field and the use of chloroform as extraction solvent heavier than water (Fig. 1-2). The system applicability for real sample analysis was demonstrated by the extraction of cationic surfactants as ion-pair with disulfine blue [6]. The configuration further allowed to keep the solvent after extraction within the tube connecting the syringe to the multiposition valve, the discharge of the sample rest and the aspiration of a cleaning solution for extract washing before measurement.

In a third work, the use of a simpler induction of the rotating magnetic field was tested. Since only mixing of sample and reagent was indented, the placement of a motor with two neodymium magnets close to the syringe allowed slow and continuous stirring of the syringe content. In this work, the syringe void was used to create a closed but size-adaptable chamber for head-space extraction of ethanol into a liquid drop of chromate reagent, which was posterior propelled into a spectrophotometric flow cell [7].

3 Results and Discussion

Experimental results and performance features of the different analyzer systems and methods will be detailed on the poster. Parameters of optimization included reagent volumes, reaction times and in the case of solvent extraction further the times of stirring assisted LLME and time required for phase separation.

In all works, high reproducibility of signals were achieved and the limit of detection values were competitive with prior works based on the same methodology but using Sequential Injection Analysis. The stirring of the syringe content allowed cleaning the syringe effectively and mixing large sample volumes of up to 4 mL with small amounts of reagent, so to avoid notable sample dilution. The complete homogenization of the syringe content further allowed to dilute the samples inside the system or to prepare the standards from one stock solution automatically and in real-time, or to perform standard addition protocols.

In all works, one analysis was possible to accomplish within a few minutes and using only a fraction of reagents compared to the manual and often lasting standard procedures. This makes in-syringe analysis a potentially green alternative to classical laboratory protocols. Since the same volume ratios and order of reagent addition can be applied as in manual procedures, straight-forward automation of standard procedures without loosing comparability can be achieved.

4 Conclusions

In-syringe analysis presents a flow technique of new features and future potential for the automation of sample treatment with the possibility of large-volume treatment within a closed analyzer system. The technique is potentially applicable for monitoring purposes and usable for the pre-treatment of biological samples such as urine, serum, or bioprocess brews.

Acknowledgement

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P44 POTENTIAL OF CONNECTING ION MOBILITY SPECTROMETRY TO LIQUID SEPARATION TECHNIQUES

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Summary
Ion mobility spectrometry (IMS) is an analytical technique based on the separation of ionized molecules with respect to their different mobilities in the gas phase. In this work the ability of corona discharge (CD) atmospheric pressure ionization source to form selective reactant ions suitable for IMS is shown. Introduction of the sample by electrospray and subsequent ionization by CD for analysis of liquids by IMS is presented, as well. Studies were carried out on the model samples of organic acids (malic, tartaric, lactic and citric) and the wine samples.

1 Introduction

IMS is an analytical technique used to characterize gaseous ions based on their mobility in a weak electric field. Main advantages of this technique include fast and reliable analysis, portability and low cost of instrumentation, as well as relatively easy manipulation. These are the main reasons why nowadays IMS is widely used in the field of explosives, chemical warfare agents and drugs detection [1].

Prior to the analysis by IMS molecules have to be ionized, which is usually achieved by radioactive sources, e.g. ^{63}Ni. In order to avoid the use of radioactive materials several alternatives have been developed, such as photoionization, electrospray ionization or CD. In comparison to most frequently used radioactive ionization technique (^{63}Ni), CD offers higher signal intensity, ability to change reactant ions, simple construction and applicability to wide range of compounds [2].

In order to optimize CD as ionization source several aspects of the ionization process have to be studied, e.g. geometry of CD, distances of electrodes and gas flow directions.

2 Experimental

CD-IMS instrument used in the experiment is described in details elsewhere [3]. This instrument is composed of: (i) an ionization chamber, where the CD is located and the ion-molecule reactions occur; (ii) a shutter grid; (iii) a drift tube, where the separation of ions occurs; and (iv) a detection unit.

Point to ring geometry was employed for introducing the sample into the IMS. Analyzed liquid samples were introduced into IMS via the stainless steel capillary heated to 470 K. This capillary was placed in front of another capillary (Fig. 1) at which the potential of 3 kV was applied. Negative CD was employed as an ionization source, while air was used as a drift gas.

Model samples of organic acids (malic, lactic, tartaric and citric) were prepared from chemicals of p.a. purity. Wine samples studied were purchased at a local store.

3 Results and Discussion

In order to analyze liquid or solid phase samples by IMS, electrospray sampling (ESS) technique (Fig. 1) was used for introduction of the sample to the capillary. Although sample was sprayed, this did not result in appearance of the charged particles, but only in transformation of the sample to vapour and droplets. Subsequently the CD reactant ions were used for ionization of the neutral particles. Great advantage of this type of sample introduction is that sample can be ionized in both negative and positive polarity.

Due to the character of analytes, negative polarity was employed for studying of organic acids. Two operation modes of CD were used for the generation of negative reactant ions: (1) reverse gas flow mode and (2) standard gas flow mode. In the first mode gas flow has the opposite direction to the direction of the ion movement in the CD gap. This mode is used for a removal of neutrals and radicals from the CD gap and for generation of "soft" reactant ions, e.g. O_2^- [3]. The second mode is characterized by the same direction of gas flow and ion movement in the CD gap and is used for generation of reactant ions, e.g. NO_3^-, $N_2O_2^-$ and $NO_3^-.HNO_3$ [2]. The selective formation of reactant ions can lead to higher sensitivity and selectivity in IMS.

Fig. 1. Photographs of ESS technique coupled with IMS.

ESS with CD-IMS was used for direct analysis of wine samples. Organic acids primary found in wine (tartaric, malic and citric), as well as some other organic acids formed during winemaking or storage process, e.g. lactic acid, were determined in this study (Fig. 2). As shown in the CD-IMS spectrum, we were able to confirm the presence of various organic acids in wine samples, e.g. malic acid.

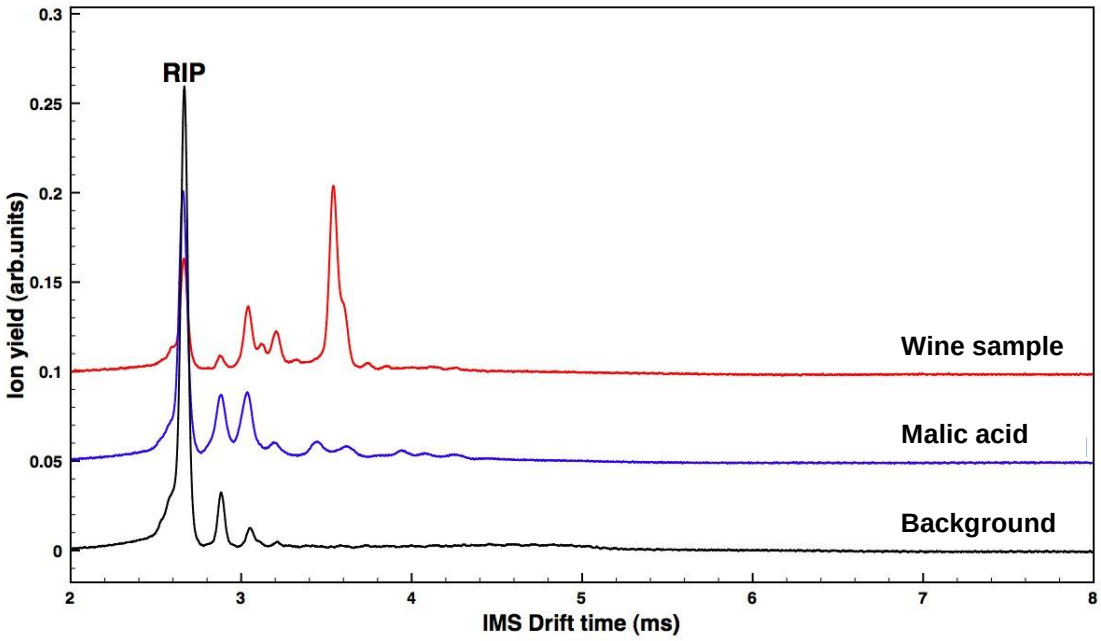

Fig. 2. CD-IMS spectrum of wine sample.

4 Conclusions

This work dealt with optimization of CD ion source for selective generation of reactant ions and its applicability to analysis of organic acids, which can be furthermore applied to another similar analytes. The sample introduction by ESS for fast analysis of liquids by IMS was presented. This type of sample introduction has the potential to be coupled with separation techniques, such as high performance liquid chromatography or capillary electrophoresis.

Acknowledgement
This work was supported by the Slovak Research and Development Agency (APVV-0259-12) and the Grant of Comenius University in Bratislava (UK/87/2014).

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P45 USE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR DETERMINATION OF SACCHARIDES AFTER THE ENZYMATIC HYDROLYSIS OF WASTE BREAD

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Summary
The main topic of our study was the effect of pH and temperature on the efficiency of enzymatic hydrolysis. For study of enzymatic hydrolysis were used spectrophotometric and HPLC methods. Optimal value of pH and temperature of liquefaction was determined spectrophotometrically by Somogyi-Nelson method. Final yields of glucose from hydrolysis and efficiency of saccharification were determined by HPLC method.

As substrate for enzymatic hydrolysis was used waste bread. It was pre-treated by grinding into small particles and then it was made to form 15% w/v suspension. For the hydrolysis have been used two enzymes. For liquefaction of the substrate we used α-amylase, and for saccharification we used glucoamylase.

It was found that optimal conditions for α-amylase were pH 6 and temperature 80 °C. Under these conditions the yield of reducing sugars was 67.08 $g \cdot L^{-1}$ (calculated to maltose). Optimal conditions for glucoamylase were pH 4.2 and temperature 60 °C. Obtained yield of glucose was 70.28 $g \cdot L^{-1}$.

1 Introduction
It is necessary to search new materials for ethanol production, due to increasing prices of raw material for ethanol production (corn, cereals, potatoes, etc.). One of the inexpensive suitable fermentation feed used in distilleries seems to be food industry

waste. Mainly waste from bakery deserves special attention. It follows that waste bread is suitable for ethanol production [1].

Enzymatic hydrolysis is one of the options of pretreatment of waste bread before fermentation. Finding optimum conditions is important to obtain higher yield of reduced carbohydrates. These are suitable for further microbial processing. Carbohydrates and other nutrients, which contains food waste, can be converted by micro-organisms into desired products, such as biofuels, functional chemicals or monomers of bioplastics [2].

The objective of this study was to find optimal pH and temperature of saccharification of waste bread. Amount of forming glucose after the gradual hydrolysis was detected by HPLC.

2 Experimental

For experiment was used wheat-rye bread (after shelf life). It was obtained from local cafe. Raw material has not been attacked by mold. Bread was crushed by mixer and chopper. It was hydrolyzed by two enzymes, one for liquefaction a second for saccharification. As liquefying enzyme was used α-amylase from Bacillus amyloliquefaciens. For saccharification was used glucoamylase from Aspergillus niger. Amount of both enzymes was 12 μL.

We chose the three different temperatures and pH values. Temperatures for α-amylase were 70 °C, 80 °C, 90 °C and values of pH 5, 6, 7. Temperatures for glucoamylase were 55 °C, 60 °C, 65 °C and values of pH 3.2, 4.2, 5.2.

Liquefaction efficiency was measured by the spectrophotometric method by the Somogyi-Nelson. This method was used for determination the amount of reducing sugars. Yields were calculated to amount of maltose. Efficiency of saccharification was measured by HPLC.

For analyse on HPLC we used as mobile phase $5 \cdot 10^{-3}$ M solution of H2SO4. Mobile phase rate flow was set to 1 mL·min-1 and was constant throughout the measurement. Time analysis of glucose calibration solution was 7 minutes. For standard solution was observed retention time of the substance. Calibration curve was based on the area of the relevant peak. Concentration of the glucose in the samples was determined by conversion via linear regression equation. It was obtained from the calibration curves.

3 Results and Discussion

It was found that the highest yield of reducing sugars during liquefaction by α-amylase was achieved at pH 6 and temperature 80 °C. The yield of reducing sugars, calculated to maltose, was 67.08 $g \cdot L^{-1}$. These conditions were used for other experiments for determination of effectivity of α-amylase.

For saccharification by glucoamylase was optimal temperature 60 °C and pH 4.2. The highest yield of glucose was 70.28 $g \cdot L^{-1}$. These conditions were chosen as ideal for saccharification by glucoamylase. Final yields were calculated from the difference between the initial and final amounts of glucose. Individual amounts of glucose are shown in Table 1.

Table 1. - Final yields of glucose from saccharifications		
Temperature	**pH**	**Concentration of glucose [g·L^{-1}]**
55°C	3,2	40,862
	4,2	45,212
	5,2	48,205
60 °C	3,2	58,879
	4,2	70,279
	5,2	54,730
65 °C	3,2	53,515
	4,2	55,916
	5,2	42,425

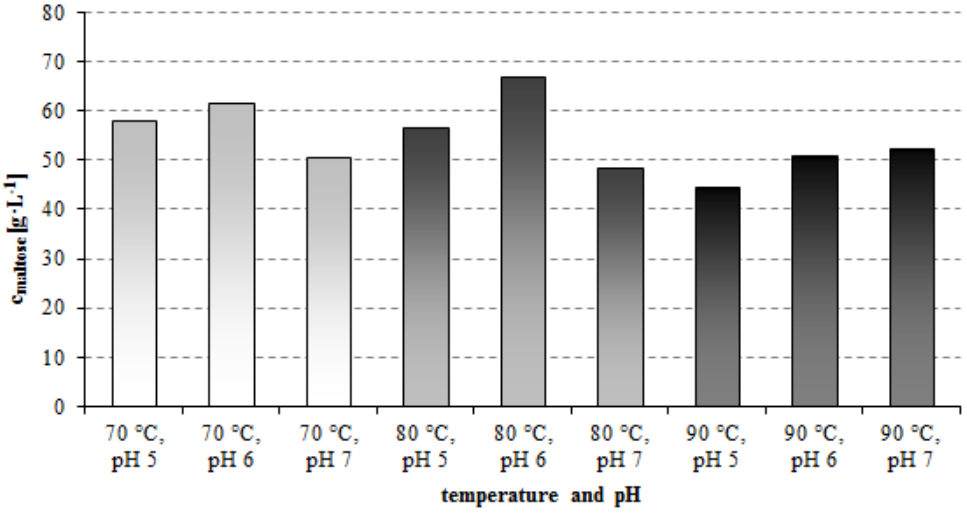

Fig. 1. Comparison obtained yields of reducing sugars during liquefaction due to temperature and pH.

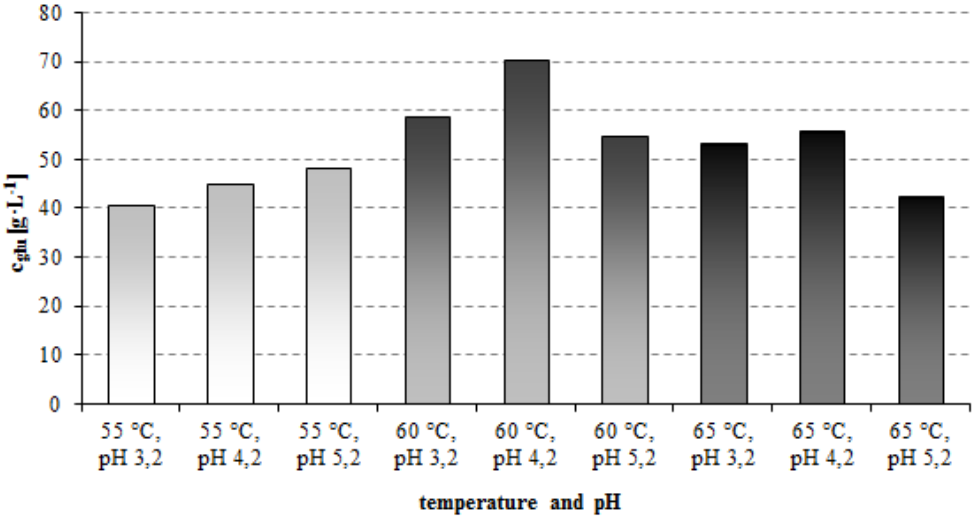

Fig. 2. Comparison obtained yields of glucose during saccharification due to temperature and pH.

4 Conclusions

We used high-performance liquid chromatography for determination efficiency of enzymatic hydrolysis of waste bread. It has been tested several different conditions of hydrolysis. Optimal conditions of the α-amylase were detected spectrophotometrically as our HPLC column does not allow detection of occurring carbohydrates. It was found that the optimal liquefaction took place at pH 6 and 80 °C.

During saccharification we found that most of the glucose was obtained at 60 ° C and pH 4.2. Applying found optimum conditions for both enzymes was obtained 70.28 $g \cdot L^{-1}$ of glucose. The acquired knowledge is important for the further processing of waste bread.

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P46 INFLUENCE OF CHROMATOGRAPHIC PARAMETERS ON HYDROPHILIC INTERACTION CHROMATOGRAPHY SEPARATION OF ALIPHATIC ACIDS ON ZWITTERIONIC STATIONARY PHASE

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1 Introduction

Small polar compounds such as aliphatic acids are often very challenging to method development, as such compounds are weakly retained in reversed-phase liquid chromatography (RP-LC), which is predominant mode used in liquid chromatography these days. Hydrophilic interaction liquid chromatography (HILIC) has been reported as an alternative to RP-LC for the analysis of polar compounds [1,2]. The term HILIC was first used by Alpert [3], who considered the main retention mechanism to be the partitioning between the mobile phase and a mobile phase layer enriched with water partially immobilized on the stationary phase. On the contrary, the retention mechanism in HILIC mode appears to be complex, involving also secondary electrostatic, hydrophobic, and hydrogen-bonding interaction dependent on the conditions used. HILIC combines polar stationary phases usually used in the normal phase liquid chromatography (NP-LC) and aqueous-organic mobile phases rich in

organic solvent typical for RP-LC. HILIC separation employs various columns such as: bare silica gel, silica-based amino-, amido-, cyano-, carbamate-, diol-, polyol-, or zwitterionic stationary phases (ZIC) [4]. In this study, ZIC stationary phase with phospohrylcholine functionaly group was used for the separation of aliphatic acids. The effect of the volume fraction of the aqueous buffer in the mobile phase and its ionic strength (molar concentration) as well as the column temperature on the retention of the test acids has been investigated.

2 Experimental

2.1 Chemicals

All analytical standard-grade aliphatic acids (malic, oxalic, L-tartaric, citric, gluconic, 2-oxoglutaric and pyruvic) were obtained from Merck (Darmstadt, Germany). Stock standard solution were obtained by dissolution of the acids in 95% aqueous acetonitril (Sigma Aldrich, St. Louis, United States) and working solutions were obtained by appropriate diluting the stock solutions in the mobile phase. Ammonium acetate (NH_4Ac) supplied by Sigma Aldrich (St. Louis, United State) were used for the preparation of mobile phase.

2.2 Apparatus

Separation of aliphatic acids was carried out with HPLC setup including a degasser, manual injector high pressure pump (ECOM, Prague, Czech Republic) connected with coularray detector (ESA 5600, Thermo Scientific). The separation was performed on silica based Merck SeQuant ZIC-cHILIC column (150 x 2,1 mm; particles 3 μm; 100 Å) with phosphrylcholine functional groups.

3 Results and Discussion

3.1 Effect of the volume fraction of the buffer solution and its molar concentration

The effect of the volume fraction (from 15 % to 30 % v/v) of the aqueous buffer solution on the retention of aliphatic acids was investigated. Because the aqueous buffer has higher elution strength than any organic solvent under HILIC conditions, increasing volume fraction of acetate buffer in the mobile phase decreased the retention of the acids. This agrees with general behavior observed in normal-phase chromatography.

Figure 1 shows the effect of increasing molar concentration of the acetate buffer on the retention of test acids on ZIC-cHILIC column. As the concentration of acetate buffer increase from 20 $mmol \cdot L^{-1}$ to 60 $mmol \cdot L^{-1}$ the retention of aliphatic acids increase and the resolution improves. The increasing retention of the acids suggests that the ion-exchange mechanism does not participate significantly on the retention of these acids. If it does, the retention would decrease proportionally to the molar concentration of the acetate buffer.

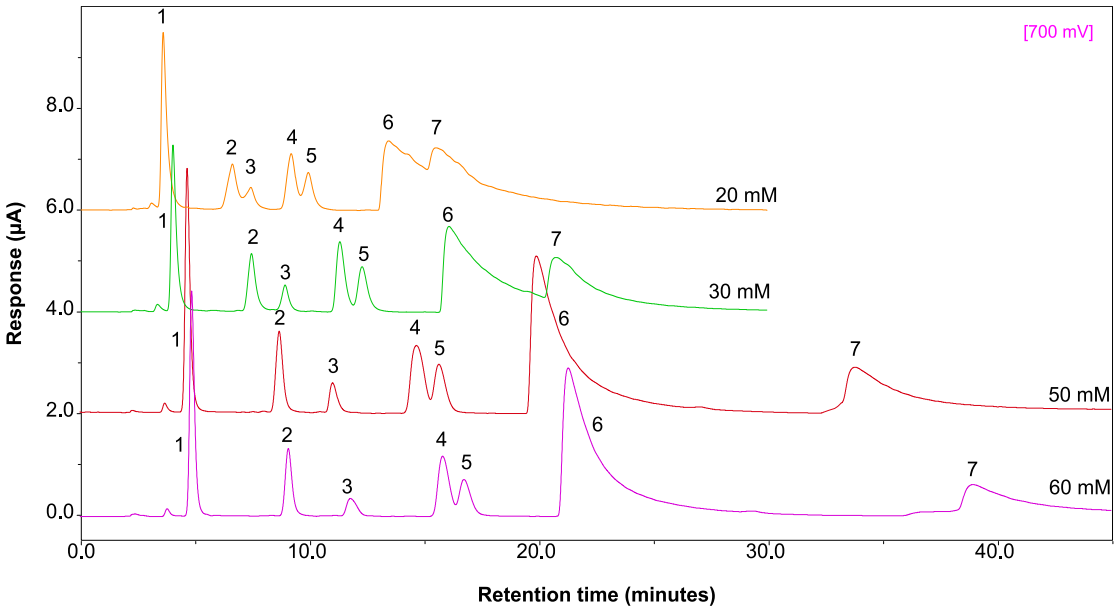

Fig. 1. Separation of aliphatic acids at different molar concentration of aqueous buffer solution Conditions: mobile phase (A) x mmol·L^{-1} NH_4Ac (B) x mmol·L^{-1} NH_4Ac : acetonitrile (5:95% v/v), temperature 40°C, flow 0,2 mL·min^{-1}, injection volume 3 µL, cell potential 700 mV. Peak identification: (1) pyruvic, (2) gluconic, (3) 2-oxoglutaric, (4) malic, (5) tartaric, (6) oxalic, (7) citric.

3.2 Temperature effect on the retention

If the retention controls a single mechanism the effect of the temperature can be describe by van't Hoff equation.

$$\ln k = -\frac{\Delta H^{\circ}}{RT} + \frac{\Delta S^{\circ}}{R} + \ln\frac{V_S}{V_M} = A_i + \frac{B_i}{T} \qquad (1)$$

where $\Delta H°$ is standard partial molar enthalpy of transfer of the solute *i* from the mobile phase to the stationary phase, $\Delta S°$ is partial molar entropy of the transfer of the solute from the mobile phase to the stationary phase, $\frac{V_S}{V_M}$ is the phase ratio, *R* is the gas constant and *T* is thermodynamic temperature [K]. A_i includes $\Delta S°$ and the phase ration and B_i is proportional to the $\Delta H°$.

If the retention controls single mechanism, the *ln k* versus *1/T* should be linear. This behaviour is common in RP-LC, where retention usually decreases with increasing temperature. In HILIC mode non-linear *ln k* versus *1/T* were observed [5-7] which may signalize changing retention mechanism. We investigated the retention of aliphatic acid in the temperature range from 25°C to 60°C. We observed deviation of the experimental data from the linearity of equation 1 for almost all tested acids except citric acid. Also, non-linear dependence *ln k* versus *1/T* for oxalic and tartaric acid was observed, which predict that retention of aliphatic acid is controlled by more than one mechanism.

4 Conclusions

In this paper the effect of chromatographic parameters on retention behavior of aliphatic acids was investigated. The dependence of the retention factors *k* on the volume fraction *φ(%)* of the aqueous buffer is linear and its decrease with increasing *φ(%).* ZIC-cHILIC provides thermal stability up to 70°C. The *ln k* of aliphatic acids shows deviations from linear dependence on *1/T*. To explain these observations, thermodynamic data for the transfer of aliphatic acids from the mobile phase to the stationary phase should be calculated applying Eq. 1 to the parameters of the Van't Hoff plots.

Acknowledgement

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P47 HILIC-RP DUAL RETENTION MECHANISM ON DIOL BASED STATIONARY PHASES

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Summary

The retention of phenolic acids was measured on the YMC Triart Diol HILIC, Luna HILIC and LiChrospher 100 Diol columns. All the columns showed dual retention mechanism; aqueous normal phase (HILIC) mechanism, appearing in the mobile phases with high content of organic solvent and reversed phase mechanism in highly aqueous mobile phases. The selectivity of the columns was evaluated by calculating the separation factors, which is the relation of the retention factor of compared compound to the least retained compound.

The correlations between the retention and the structure of sample compounds were investigated using Abraham linear free energy relationship model. The retention in the HILIC mode is mostly controlled by proton-donor interactions of the stationary phase with sample solutes. The highest retention, selectivity and efficiency are the highest on the LiChropsher column, nevertheless, on the YMC column is the efficiency and selectivity almost comparable.

1 Introduction

The principle of HILIC consists in adsorption of water from organic/aqueous mobile phase (the range of content of water is often around 2-20%) onto the surface of polar stationary phase, where it creates a diffuse layer. The retention is probably combined of two major mechanisms, partition of the solute into the water-rich layer and adsorption onto the surface of polar stationary phase [1]. HPLC systems, where the stationary phase is moderately or strongly polar and mobile phase is composed of polar (water) and less polar solvent (ACN, methanol) show dual retention mechanism, which can be described by eq. (1) [2]:

$$\log k = a_6 + m_{RP} \cdot \varphi_{H2O} - m_{HILIC} \cdot \log(1 + b \cdot \varphi_{H2O}) \quad (1)$$

One part of the work has focused on the study of the dual retention mechanism on three various forms of diol bonded phases. As the model compounds, phenolic acids were used.

Next part of the work has focused on the influence of various polar interactions between a sample and a stationary phase on the retention of the sample. The relation can be described by linear free energy relationship (LFER) model. The LFER model employs multiple correlations between the retention (log k) and so-called solvatochromic parameters, characterizing the solubility and solvation of the solute and the stationary phase [3]:

$$\log k = c + v \cdot V + s \cdot S + a \cdot A + b \cdot B \quad (2)$$

where V, S, A, B and are the structural descriptors characterizing the sample: the volume of the solute, V, dipole-dipole interactions, S, hydrogen bonding acidity, A, and hydrogen bonding basicity, B. The coefficients v, s, a, and b of Eq. (2) provide a measure of the response of the system stationary/mobile phase to the selective properties of analytes.

2 Experimental

The experiments were performed at the flow rate of mobile phase set to 0.2 mL/min for the YMC and Luna columns, 0.6 mL/min for the LiChrospher column. The temperature was set to 40°C. The retention factors were calculated from experimental retention times of each compound and column hold up time for various volume fractions of H_2O in mobile phase. The retention was measured at five various volume fractions of buffered water (φ_{H2O}) in mobile phase in the HILIC mode and at four/ five various volume fractions in the RP mode.

3 Results and discussion

The retention of phenolic acids was investigated over the full mobile phase composition range (2-95% 10 mM acetate buffer in mobile phase-φ_{H2O}). All Plots of retention factor (k) dependence on content of water (10mM NH_4Ac, 0.1% HCOOH) in mobile phase (φ_{H2O}) showed "U-shape". The HILIC retention mechanism predominates in the range of 2% to ~ 50% of buffered water in the mobile phase (depends on a type of a stationary phase and a solute) and with the content of buffered water in mobile phase more than ~50%, the RP mechanism begins to control the retention. The volume fraction of water, φ_{H2O} corresponding to the minimum of the U-plot, which indicates the transition from the HILIC to the RP mechanisms, was the highest on the LiChrospher Diol column. In the HILIC mode, the relative retention of phenolic acids on the LiChrospher column is significantly higher in comparison to Luna and YMC columns. Luna HILIC shows the lowest retention due to its lower polarity caused by the presence of oxyethylene groups on the surface.

Regarding Abraham LFER model, the parameters v, s, a, b were obtained as a result of a multiple linear regression analysis fitting the experimental retention factors, k, to the LFER model described by eq. 2. The highest values, thus the greatest effect on the retention of phenolic acids had the parameters v and b.

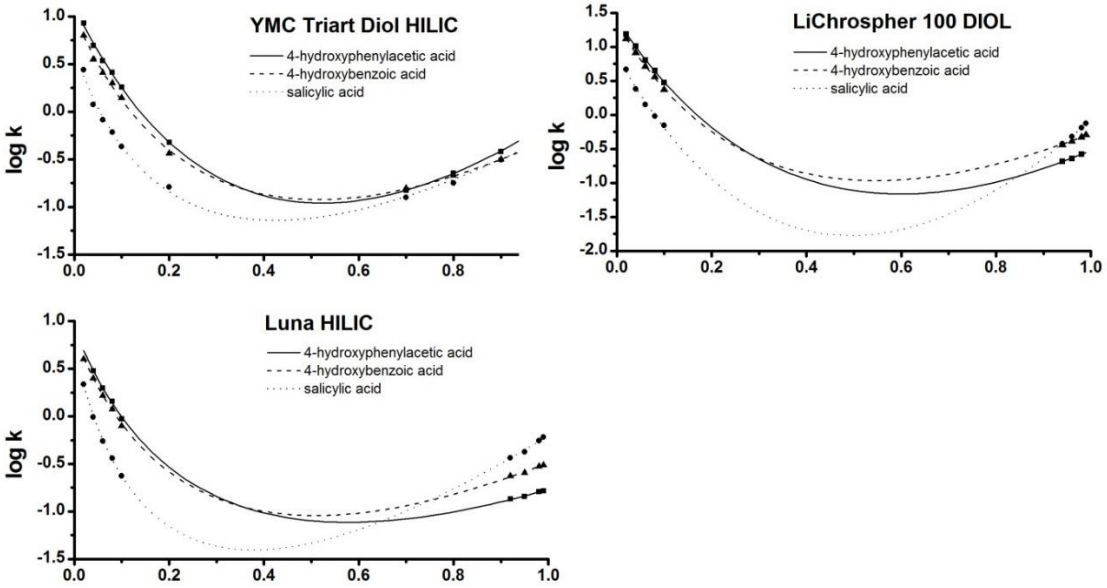

Fig. 1. Effect of the volume fraction of aqueous buffer, φH2O on the retention factor k of three phenolic acids on three diol based stationary phases. Points-experimental data, lines-best fit plots of eq. (1).

The selectivity was evaluated by calculating the separation factors, α, which signify the relation of the retention factors of compared phenolic acids to the retention factor of the least retained compound, in this case salicylic acid. In the HILIC mode, the separation factors of phenolic acids are the highest on the LiChrospher column (nevertheless, almost comparable to the YMC column). Calculating the height

equivalent of theoretical plate from the values of number of theoretical plates and length of the column gave us information that the highest efficiency was on the LiChrospher column.

4 Conclusions

The LiChrospher Diol column shows the best properties for the separation of small polar compounds; the highest retention, selectivity and efficiency in the HILIC mode. In addition the operability in broad range of the composition of the mobile phase under HILIC conditions is achieved (the most polar 4-hydroxyphenylacetic acid has the minimum of the U-plot at the 60 % of the buffered water in the mobile phase).

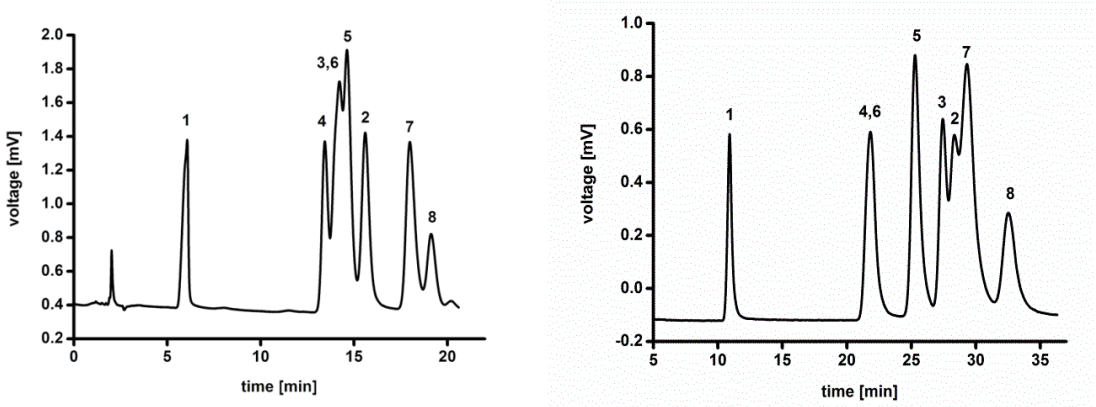

Fig. 2. Separation of phenolic acids on the YMC Triart Diol HILIC (left), (Conditions**:** 10 mM NH4Ac in O - 3%, 10 mM NH4Ac in ACN - 97%, flow rate 0.2 mL/min, temperature 40 °C) and LiChrospher Diol (right). (Conditions**:** 10 mM NH4Ac in - 2%, 10 mM NH4Ac in ACN - 98%, flow rate 0.6 mL/min, temperature 40 °C). 1. salicylic acid, 2. coumaric acid, 3. 4-hydroxybenzoic acid, 4. ferulic acid, 5. vanilic acid, 6. sinapic acid, 7. syringic acid, 8. 4-hydroxyphenylacetic acid.

An alternative is using the new developed column YMC Triart Diol HILIC, which has the almost comparable selectivity and efficiency. An advantage of use of the YMC column are the cheaper analyses due to the lower flow rate of the mobile phase and achieving the similar separations in significantly lower retention times.

The retention in the HILIC mode is controlled by proton donor properties of the stationary phase. These interactions are the strongest on the LiChrospher column.

Acknowledgement

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P48 OPTIMIZATION OF ANALYTICAL METHODS FOR THE DETERMINATION OF RESIDUES OF SELECTED PHARMACEUTICALS IN THE AQUATIC ENVIRONMENT

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Summary
The present study deals with the determination of residues of selected antibiotics from the group of sulfonamides in water ecosystem. The target analytes included ten sulfonamides: sulfaguanidine, sulfadiazine, sulfathiazole, sulfapyridine, sulfamerazine, sulfadimidine, sulfamethoxazole, sulfadoxine, sulfaclozine and sulfadimethoxine. The optimized methods should be applied for the matrices as surface or waste water and bottom river sediment. The attention was first focused on optimization of the method for identification and quantification of selected sulfonamides using high-performance liquid chromatography coupled with diode array detector (HPLC/DAD). The methods of solid phase extraction (SPE) and pressurized solvent extraction (PSE) were optimized for extraction, pre-concentration and purification of the matrices.

1 Introduction

Antibiotics, including sulfonamides, are a large group of pharmaceuticals whose consumption in both human and veterinary medicine is still growing. In both human and animal body they are metabolized and excreted in the urine or faeces, while 30 to 90 % of them can be excreted in active form. In this way, directly or via use of livestock manure as a fertilizer or via wastewater treatment plant, antibiotics in their original or metabolized form can enter the environment. There they can accumulate, persist and thus negatively affect ecosystems, including water ecosystem, where there are toxic especially to aquatic organisms. The occurrence of residual antibiotics in the environment then results in an increased incidence of resistant bacteria, which may in future become a potential threat to human health. From these reasons the monitoring of residual antibiotics in the aquatic environment is necessary, and reliable pre-analytical and analytical methods are essential for precise results.

2 Experimental

The efficiency of particular methods was verified using analytical standards of selected pharmaceuticals dissolved in methanol, while a mixed standard of all 10

sulfonamides (concentration of 25 mg·ml^{-1} for aqueous samples and 50 mg·ml^{-1} for solid matrix) was prepared.

Optimized parameters of the HPLC/DAD analytical method were as follows:
- solvents for mobile phase: acetonitrile, methanol, 0.01M HCOOH/water;
- gradient elution: various ratios of the two mobile phases varying in time;
- various mobile phase flow rate: in the range from 0.150 to 0.250 ml/min, including the test of flow gradient;
- column temperature: in the range from 15 to 35 °C, including the test of temperature gradient.

Spiked pure Milli-Q water was used as a model water sample. The method of solid phase extraction (SPE) was used to extract the sulfonamides from spiked water samples while the optimization and selection of the most suitable method was verified using several different procedures. Recovery of each method was calculated based on the observed concentrations.

Table 1. SPE procedures used for water extraction.

procedure no.	1	2	3	4	5	
column	Supel-Select HLB	Supel-Select HLB	Supel-Select HLB	PEP Plus	PEP Plus	
conditioning	MetOH - 2 ml DI water - 2 ml	MetOH - 2 ml DI water - 2 ml	0,1M HCOOH in MetOH - 2 ml 5% MetOH - 2 ml	0,1M HCOOH in MetOH - 2 ml 5% MetOH - 2 ml	MetOH - 5 ml DI water pH 4 (using HCl) - 5 ml	WASTE CONTAINER
sample loading	100 ml, flow rate 1-2 drops/sec.	100 ml, flow rate 1-2 drops/sec.	100 ml, flow rate 1-2 drops/sec.	100 ml, flow rate 1-2 drops/sec.	100 ml, flow rate max. 4 ml/min.	
washing	10% MetOH-2 ml	5% ACN - 2 ml	5% MetOH - 2 ml	5% MetOH - 2 ml	2% MetOH - 2 ml	
drying	5-10min. vacuum	5-10min. vacuum	5-10min. vacuum	5-10min. vacuum	5-10min. vacuum	
elution	MetOH:ACN (1:1) 5 ml	MetOH:ACN (1:1) 5 ml	0,1M HCOOH in MetOH 5 ml	0,1M HCOOH in MetOH 5 ml	MetOH - 2 x 5 ml two elutions	COLLECT. CONT.

MetOH = methanol, DI = deionized water, ACN = acetonitrile, HCOOH =formic acid, HCl = hydrochloric acid

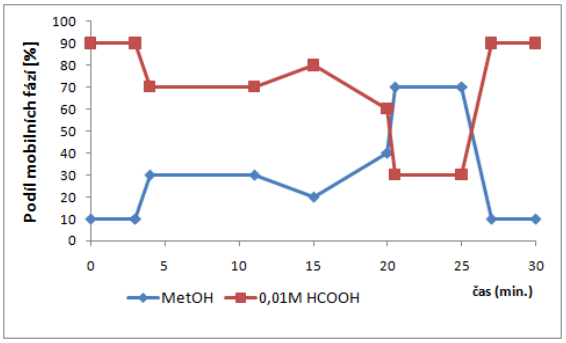
Spiked sea sand was used as a model sediment sample. The method of pressurized solvent extraction (PSE) was used to extract the sulfonamides from spiked model matrix and SPE was used for purification and pre-concentration of the extracts.
Optimized parameters of the PSE extraction method were as follows:
- temperature: 40 °C, 50 °C, 60 °C;
- pressure: 6 MPa, 7 MPa, 8 MPa; (in various combinations).

Optimized parameters of the SPE purification method were as follows:
- type of SPE cartridge: ENVI-18, Cleanert PEP Plus, Waters Oasis HLB;
- solvents for conditioning, washing and elution.

Recovery of each method was calculated based on the observed concentrations.

3 Results and Discussion

3.1 HPLC/DAD



Graph 1. Mobile phase gradient.

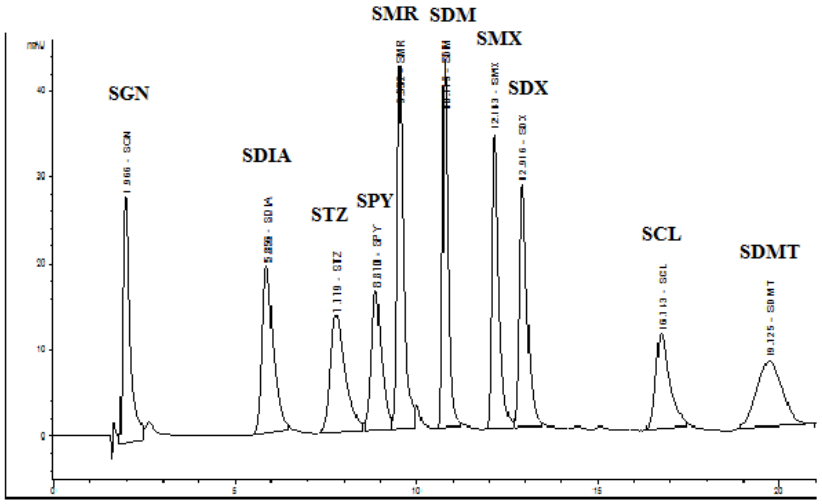


Fig. 1. Chromatogram of mixed standard of 10 sulfonamides.

Table 2. Selected final conditions of HPLC/DAD analysis.

Column type	ZORBAX Eclipse XDB-C8 2.1 x 150 mm; 3.5 µm
Injection volume	1 µL
Mobile phase solvents	0.01M HCOOH/water methanol
Mobile phase flow rate	0.250 mL/min
Column temperature	35 °C
Detection wavelength	270 nm
Stop Time	30 min
Post Time	10 min

3.2 Water – SPE; Sediment – SPE + PSE

As the optimal the method no. 2 (Table 1) was evaluated, while the highest recoveries were achieved in all monitored sulfonamides. Recovery of this method ranged from 62.7 % (sulfathiazole) to 97.4 % (sulfadimethoxin); the mean recovery for all 10 sulfonamides was 79 %.

Recoveries of the method selected for isolation of sulfonamides from solid matrix ranged from 46.0 % (sulfathiazole) to 95.8 % (sulfadimidine) regarding individual sulfonamides. The mean recovery for all 10 sulfonamides was 78.8 %.

Table 3. Selected parameters for isolation of analytes from water samples.

SPE cartridge type	Supel-Select HLB SPE (6 mL/200 mg)
Conditioning	2 mL methanol 2 mL deionized water
Sample application	500 mL water sample flow rate 1-2 drops/sec.
Sorbent washing	2 mL 5% acetonitrile
Drying	10 min. air flow
Elution	5 mL methanol:acetonitrile (1:1) flow rate 1-2 drops/sec.

Table 4. Selected parameters for isolation of analytes from solid matrix.

PSE	**Extraction solvent**	methanol
	Temperature	40 °C
	Pressure	6 MPa
	Cycles	2
SPE	**SPE cartridge type**	Cleanert PEP Plus SPE (6 mL/500 mg)
	Conditioning	2 mL 0.1M HCOOH/methanol 2 mL 5% methanol
	Sample application	50 mL of extract flow rate 1-2 drops/sec.
	Sorbent washing	2 mL 5% methanol
	Drying	10 min. air flow
	Elution	4 mL 0.1M HCOOH/methanol flow rate 1-2 drops/sec.

4 Conclusions

Considering all the obtained recoveries of the individual optimized methods we can concluded that the selected analytical methods are suitable for the extraction, isolation and determination of sulfonamide antibiotics from selected matrices.

Acknowledgement

The work has been supported by the financial contribution of the projects IGA VFU Brno no. 22/2012/FVHE and FRVŠ MŠMT of the Czech Republic no. 2180/2011/G4.

P49 COMPUTER ASSISTED DEVELOPMENT OF A PNEUMATIC ELECTROSPRAY NEBULIZER

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Summary
In this study we demonstrate how computer simulations can assist the development of a novel microfabricated pneumatic electrospray nebulizer. The microdevice under consideration is designed for electrospray mass spectrometry interfacing without the need to fabricate an electrospray needle. This new interfacing strategy requires comprehensive evaluation of the differently designed chip layouts. The flow evaluation was supported by computational fluid dynamics simulations. It was proved with experimental validation of the simulation results that the developed models were practical tools for the numerical investigation of the separations-ESI-MS coupling.

1 Introduction

Electrospray ionization (ESI) interface is a key component for on-line coupling of separations with mass spectrometry (MS). As regulatory agencies require orthogonal characterization methods for biotherapeutics (both innovator and biosimilars), capillary electrophoresis (CE) has emerged in bioanalytics. However, hyphenation of CE with ESI-MS is not straightforward due to their differences in current levels [2] (microampers in CE vs. nanoampers in ESI) and flow rates (often no flow in neutrally coated CE capillaries). Microfabricated interfaces, as a minor group of interface categories, address the issue of handling limited amount of samples, sensitivity and speed, and avoid cross-contamination. To speed up the design process, computational fluid dynamic (CFD) modeling techniques could be used to propose new microfabricated interfaces at low cost with a minimum number of actual experiments. They are especially useful to shed light on the various phenomena occurring in the microfluidic environment. For example, the microchannels in the interface systems have very high surface to volume ratio, therefore fluid flow could exhibit unusual behavior that is different than in the macroscopic world.

In this paper we report on the design, microfabrication and test of a novel, integrated liquid junction based pneumatic nebulizer, suitable for ESI-MS analysis of biological macromolecules with special emphasis on the modeling strategy.

2 Numerical experiment

The CFD models for the simulation of the flow filed were based on a one phase turbulent form of the Navier-Stokes equation. Figure 1 depicts the schematic representation of the device and its surrounding along with the modeled domain of interest. The sheath gas (nitrogen) entered the nebulizer channels at 6 bars and 293 K. Under these conditions of channels the gas exited the chip at a very high velocity, dragging the analyte from the edge of the nearby separation channel. Numerical simulations carried out focusing only on the gas phase flow. The calculations were done in 2D mirror symmetric mode in order to reduce the computational demand. Based on the theoretical and experimental finding of Grym et al. [3], which refers to a similar interface device, the performance of the electrospray was considered mostly dependent on the flow characteristic of the nebulizer, therefore, the effect of electrohydrodynamics due to the applied electric field and the liquid phase (flowing in the separation channel) was neglected. According to the calculated Reynolds number (Re ≈ 9000), which indicated the flow characteristic, the fluid dynamics was rather turbulent.

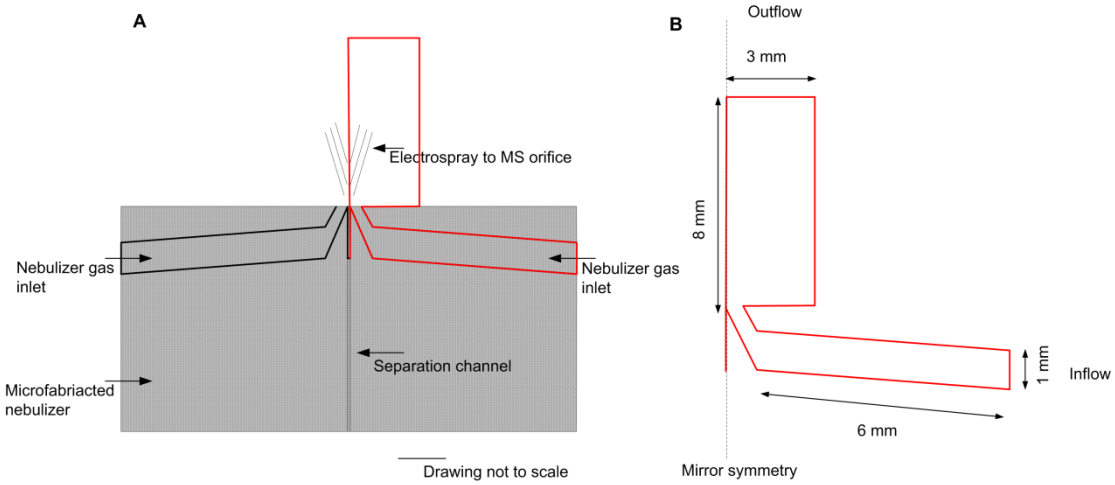

Fig. 1. Schematic representation of the spray device and the modeled domain of interest. A) The gray part is the body of the micronebulizer (converging design). The red part is the modeled domain covering the chip and the gap between the nebulizer and the MS orifice, this is where the nebulizer gas flows through. B) the simulated domain (converging design) and its dimensions.

Since this problem is highly nonlinear, the meshing (Delaunay free triangular method) was performed many times with different sizes to obtain grid-independent data [4]. At close to the nebulizer gas outlet point of the chip, the mesh was set denser in order to properly resolve the most intensive change of the flow and pressure fields. Stationer solver of commercially available software COMSOL Multiphysics version 4.3.0.151 was used to obtain time invariant solution.

3 Results and Discussion

The 2D meshed geometry consisted of 506222 triangular geometry elements together with Cartesian units in the boundary layers. Figure 2 shows the calculated velocity fields.

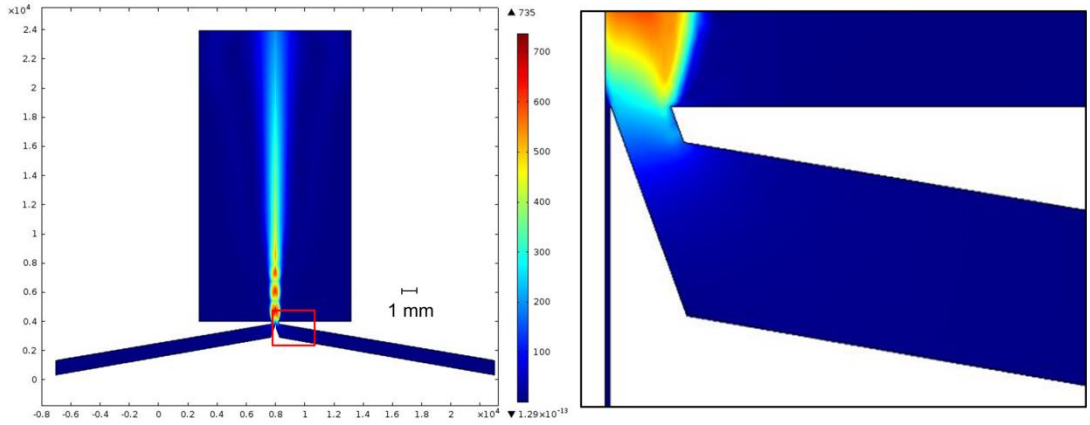

Fig. 2. Simulated flow field of gas exiting the chip. The warmer the color the higher the velocity (m/s). The right panel is the magnified view of red rectangle indicated part.

The resulted flow velocity field was not continuous. Free jet with oblique shockwaves were formed due to the applied high input pressure of the nebulizer gas. The presence of expansion – compression fans were not surprising, considering the current operation conditions, where the velocity was much above the speed of sound at 293 K. This is further verified by its independence form the applied mesh resolution (maximum discrete element size was varied from 5 to 100 μm).

The most promising microdevices were prototyped in glass using conventional photolithography, followed by wet chemical etching and thermal bonding. However, details of the applied microfabrication and MS test measurements are out of the scope of this presentation (see [5] further information). The simulated flow filed was compared with experimental spray plume perpendicularly illuminated by an expanded 532 nm laser beam. The signal was recorded by a CCD camera (Figure 3). The width of the observed plume was in good agreement with the simulated one, which was around 1 mm (measured at 3 mm from the exiting hole).

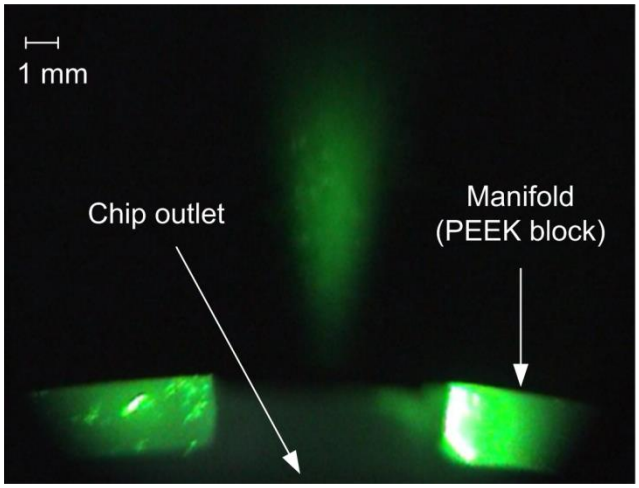

Fig. 3. A representative frame of the CCD recorded movie investigating the nebulizer layouts [5]. The electrosprayed beam (applying 3.5 kV spray voltage) was illuminated by a 532 nm NdYAG laser.

4 Conclusions

A novel, tip-less microfabricated ESI-MS interface was investigated with numerical simulations. The described modeling concept, based on the reported results proved as a practical tool for the further simplification of microfabricated separation-ESI interfaces such as the demonstrated sonic nebulizer devices.

Acknowledgement

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P50 DETERMINATION OF TESTOSTERONE AND ITS 5 METABOLITES IN THE INCUBATION MEDIA AS A MEASURE OF CYTOCHROME P450 METABOLIC ACTIVITY

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Summary
The innovative HPLC method for the determination of testosterone and its five metabolites in incubation medium with rat liver microsomes is reported. The method utilizes liquid-liquid extraction procedure, with use of internal standard prednisone. Analytes are eluted within 18.7 min. The method enables reliable determination of testosterone, 7α-hydroxytestosterone, 6β-hydroxytestosterone, 16α-hydroxytestosterone, 16β-hydroxytestosterone, 2β-hydroxytestosterone with LOD in the range 1.9-3.9 nmol/L.

1 Introduction

Testosterone is widely used substrate for the metabolic activity assessment of multiple cytochrome P450 enzymes in a single run [1-5]. Limited LC methods for determination of testosterone and its hydroxylated metabolites have been published to date [1-3, 6].

Due to the same molecular mass and polarity of the metabolites, careful separation is necessary for accurate quantitation of the metabolites. Previously published methods which trace mostly 6β-hydroxytestosterone [7] or need LC system enabling column switching [1], are quite time-consuming and thus are inappropriate for routine usage in evaluation of tenths or hundreds of samples in single experiment. Other methods enabling determination of more hydroxylated metabolites of testosterone were also published [3], but our method brings several advantages over the published HPLC-UV/DAD methods.

2 Experimental

2.1 Chemicals, samples

Testosterone, 7α-hydroxytestosterone (7α-T), 6β-hydroxytestosterone (6β-T), 16α-hydroxytestosterone (16α-T), 16β-hydroxytestosterone (16β-T) and 2β-hydroxytestosterone (2β-T) were purchased from Steraloids (Newport, Rhode Island, USA), prednisone was purchased from SigmaAldrich (Darmstadt, Germany), dichlormethane and acetonitrile were of HPLC grade and were purchased from Scharlau Chemie S.A. (Madrid, Spain). Rat liver microsomes (RLM) were obtained from adult control Wistar albino rats; the animals were sacrificed according to the

protocol of the study and were approved by Committee for Animal Welfare at Masaryk University.

2.2 Equipment

The HPLC system consisted of a LC-10ADvp gradient pump with DGU-14A degasser, SIL10ADvp autosampler, CTO10ACvp column oven, SPDM10 Avp DAD detector and SCL10Avp system controller. Data from the detector were collected and analyzed using LabSolution 1.03 SP3 software (Shimadzu, Kyoto, Japan). A PFP Kinetex chromatographic column (150 × 4.6 mm; 2.6 μm) (Phenomenex, Torrance, CA, USA) was used for the separation.

2.3 Sample preparation

The internal standard prednisone (2.5 mM, 50 μL) was added to the the samples from RLM incubations (1 mL) with the probe substrate testosterone (100 μM), 50 mM KH_2PO_4, Na_2HPO_4, 1,2 μM EDTA, 6 mM $MgCl_2$, 1.2 mM NADP, 6 mM glucose 6-phosphate and glucose 6-phosphate dehydrogenase (1.6 IU/mL). The samples were extracted with 5 mL of dichlormethane by horizontal vortexing for 10 minutes at 1400 rpm. Organic fraction (4.5 mL) was decanted and evaporated at 30°C under gentle stream of nitrogen. The evaporated extracts were dissolved in 250 μL of mobile phase (water:methanol:acetonitrile 56:40:4; v/v/v) and samples were kept at 4°C until administration.

2.4 Chromatographic conditions

The analytes were separated at 45 °C using gradient elution as follows: min. 0: water:methanol:acetonitril 56:40:4; to water:methanol:acetonitrile 44.5:50:6.5 in the 8th min., then isocratically till 16. min and then the composition of mobile phase was changed to original values water:methanol:acetonitrile 44.5:50:6.5; v/v/v and remained till min 24. to equilibrate the column for another analysis. The mobile phase flow was maintained at 0.75 mL/min and analytes were detected at 245 nm.

3 Results

The retention times of 7α-hydroxytestosterone, 6β-hydroxytestosterone, prednisone (internal standard), 16α-hydroxytestosterone, 16β-hydroxytestosterone, 2β-hydroxytestosterone and testosterone were 6.9, 7.5, 9.6, 10.5, 11.8, 13.1 and 18.7 min, respectively. Figure 1 presents a typical chromatogram of analysis of blank RLM incubation medium spiked with standards (5 μM), Figure 2 presents a typical chromatogram of sample of RLM containing medium incubated for 20 minutes with 100 μM testosterone at 37°C. Basic parameters from the method validation are reported in the Table 1.

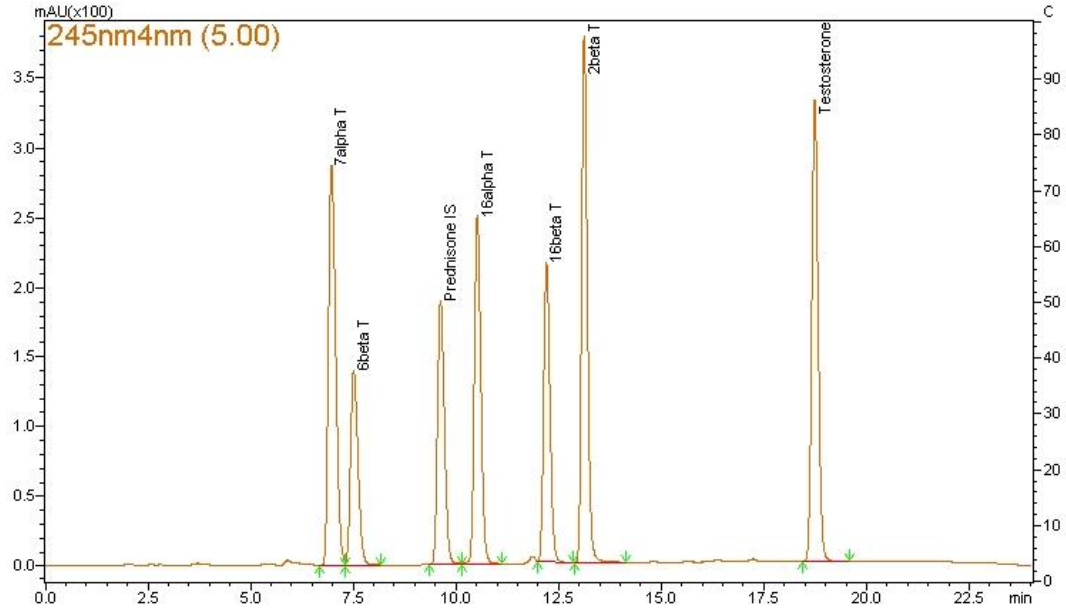

Fig. 1. Typical chromatogram of blank incubation media with RLM spiked with standards and internal standard (prednisone).

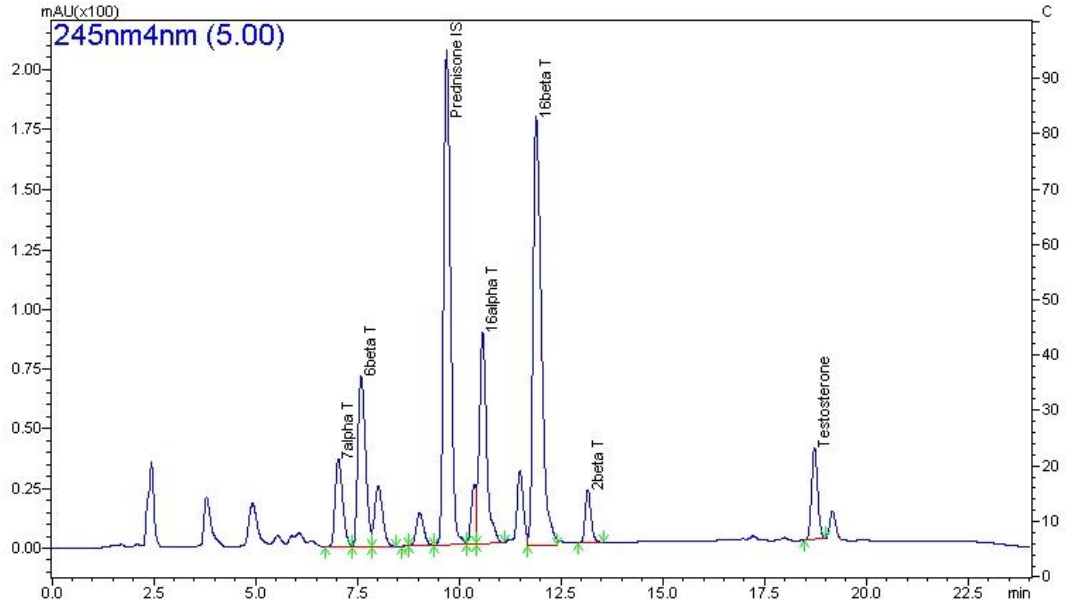

Fig. 2. Typical chromatogram of incubation medium with RLM incubated for 20 minutes with 100 nmol of testosterone at 37°C.

Table 1. Basic characteristics of the method validation.

	Linearity range (μM)	Linearity determination coeff. (R^2)	LOD (s/n=3) nM	Extraction recovery at 5 μM (%)	Intra-day precission at 5 μM (RSD)	Intra-day precission at 25 μM (RSD)
7α-T	0.026-821	> 0.999	1.92	82.8	1.12	1.60
6β-T	0.021-722	> 0.999	3.91	82.4	1.36	0.57
Prednisone (I.S.)	0.018-558	> 0.999	2.88	81.3	--	--
16α-T	0.027-854	> 0.999	2.27	85.1	0.79	1.18
16β-T	0.021-656	> 0.999	2.79	85.5	0.78	0.67
2β-T	0.022-690	> 0.999	1.64	86.1	0.95	2.67
Testosterone	0.023-745	> 0.999	1.99	90.7	1.34	1.74

4 Conclusion

Innovative HPLC- DAD method for simultaneous determination of testosterone and its five metabolites in incubation medium for RLM incubations was developed and validated. Presented method utilizes prednisone as an internal standard to ensure precision and accuracy of extraction and HPLC analysis. This method may be useful in preclinical research of various influences on cytochrome P450 metabolic activity, including drug-drug interactions with use of in vitro RLM incubations.

Acknowledgements

This work was supported by "CEITEC - Central European Institute of Technology" (CZ.1.05/1.1.00/02.0068) from European Regional Development Fund and the Project of specific research at the Masaryk University (MUNI/A/0886/2013).

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P51 METABOLITE PROFILING OF SERUM FROM HORSES WITH ATYPICAL MYOPATHY

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1 Introduction

Equine multiple acyl-CoA dehydrogenase deficiency also known as atypical myopathy (AM), is a highly fatal muscle disease of grazing horses. This syndrome is accompanied by muscular weakness, stiffness, acute myonecrosis and myoglobinuria, which in at least 75% of cases leads to death within 72 h. It is caused by ingestion of *Acer negundo* seeds containing hypoglycin A, whose active metabolite, the methylenecyclopropylacetic acid, is responsible for an irreversible inhibition of short and medium chain acyl-CoA dehydrogenases [1, 2].

The aim of this work was to compare the serum metabolomic profile of horses suffering from AM and controls.

2 Experimental

10 serum samples from horses with AM and 12 control serum were collected. An analysis was performed using high performance liquid chromatography (Ultimate 3000, Dionex) with aminopropyl column (Luna 3 μm NH2, 2 x 100 mm, Phenomenex) coupled to tandem mass spectrometry (QTRAP 5500, AB Sciex). The metabolites were detected by multiple reaction monitoring (MRM) in both positive and negative mode, quantified using MultiQuant 3.0 software and statistically evaluated in R programme language with statistics packages. Data were processed by unsupervised (Principal component analysis - PCA) and supervised (Discriminant function analysis - DFA, Orthogonal partial least squares discriminant analysis - OPLS-DA) multivariate analysis.

3 Results and Discussion

A total of 168 metabolites was analyzed in equine serum using the MRM transitions and retention times. The results from supervised and unsupervised statistical methods show obvious separation of group with AM and control group. The biggest

differences were found in levels of glycine conjugates (isobutyrylglycine, hexanoylglycine, suberylglycine, phenylpropionylglycine), acylcarnitines (C5-C18) and purine metabolites (inosine, hypoxanthine, adenine, adenosine).

4 Conclusions

A part of these observations well corresponds with previously published results [2, 3], but until now there was no study dealing with the overall metabolomic profile performed.

Acknowledgement

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P52 *N*-GLYCAN GLUCOSE UNIT (GU) DATABASE FOR CAPILLARY GEL ELECTROPHORESIS

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Summary

In this work we report on a *N*-glycan glucose unit (GU) database for 8-aminonaphtalene-1,3,6-trisulfonic acid labeled carbohydrates that was generated by a vertical capillary gel electrophoresis system with light-emitting diode induced fluorescent detection (CGE-LedIF). At this stage the database contains information on 25 glycans with glucose unit (GU) values, structural description and compared data with *N*-glycans released from glycoproteins.

1 Introduction

In the post-genomic area, one of the next major challenges is to identify and understand post-translational modifications, especially glycosylation. Glycosylation plays a crucial role in protein folding, interaction, mobility and stability as well as in

signal transduction. Thus, gylcosylation is involved in the normal functioning of the cell and in the development of disease by regulating protein aktivity [1]. The most frequently used techniques for carbohydrate analysis are high performance liquid chromatography (HPLC), capillary electrophoresis (CE) and mass spectrometry (MS) [2]. Capillary gel electrophoresis (CGE) is quickly becoming a more and more widely accepted separation and chararacterization tool for the analysis of glycan moieties of biotherapeutics. CGE is an easy-to-use automated system, also providing high resolving power, excellent detection sensitivity as well as rapid analysis time for carbohydrate profiling [3].

2 Experimental

Glycoprotein digestions were performed using the Glykoprep digestion module (ProZyme, Hayward, CA). The released glycans were dried in centrifugal vacuum evaporator. The dried sugars, maltodextrin ladder, maltose and carbohydrate standards were fluorescently labeled via reductive amination by the addition of 6.0 µL of 0.1 M ANTS in 15% v/v acetic acid and 2.0 µL of 1 M sodium cyanoborohydride in tetrahydrofuran at 37ºC overnight. The upper bracketing standard (maltose) was labeled by addition of 6.0 µL of 0.1 M ANSA in 15% v/v acetic acid and 2.0 µL of 1 M sodium cyanoborohydride in tetrahydrofuran at 37ºC overnight. After the unreacted fluorescent dye was removed by ProZyme cleanup cartridges (ProZyme). Briefly, the binding step was performed in 200 µL 1x cleanup solution to each glycan sample, following by washing off the unreacted dye from the cartridges with 200 µL 1x cleanup solution, and finally the elution of glycans by water. Capillary electrophoresis profiling of the ANTS-labeled *N*-Glycans was performed in a single-channel capillary cartridge GL1000 Glycan Analyzer (BiOptic, New Taipei City, Taiwan). The separations were accomplished in a 11.5 cm effective length (15.5 cm total length) vertical capillary column. The detecion system utilized a LED source of 350-390 nm range to match the ANTS excitation/emission spectra.

3 Results and Discussion

As shown in Figure 1, the *N*-glycans were released from glycoproteins *(1)* with PNGase F endoglycosidase enzyme *(2)*. The released glycans and the glycan standards were labeled via reductive amination using 8-aminonaphtalene-1,3,6-trisulfonic acid *(3)* followed by removal of the unreacted fluorescent dye *(4)*. Finally, the labeled sugar structures were separated by a capillary gel electrophoresis *(5)*. After the migration time normalization process by the bracketing standards, all CGE electropherograms were compared to the corresponding ANTS-labeled malto-oligosaccharide ladder for proper glucose unit value assignment to generate database entries *(6)*.

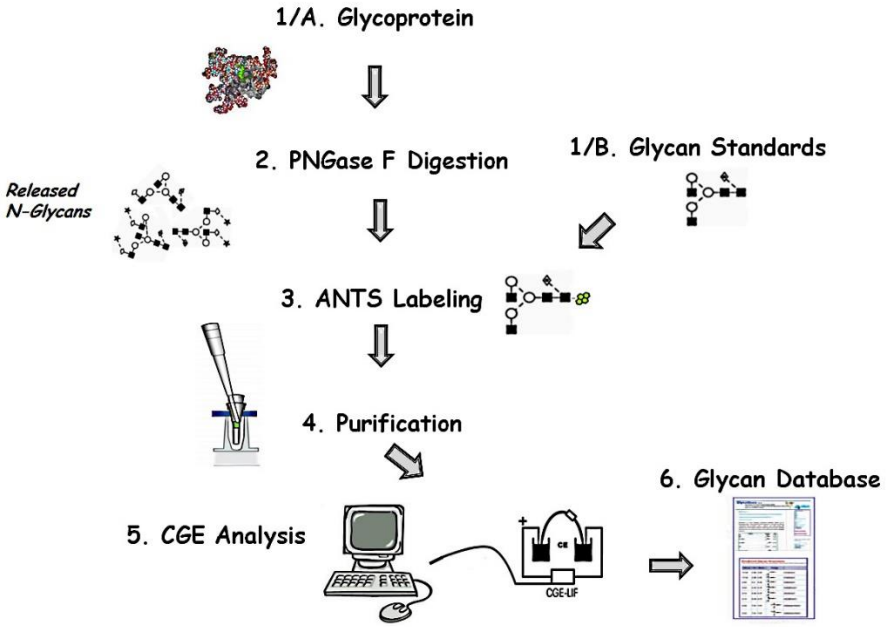

Fig. 1. An overview of the experimental strategy used for releasing, ANTS-labeling and sequencing *N*-Glycans by a novel CGE system.

4 Conclusions

At this time, 25 oligosaccharide standards represent this set of glucose unit database for 8-aminonaphtalene-1,3,6-trisulfonic acid labeled sugars. In addition, validation of the generated glucose unit values was verified with *N*-glycans released from glycoproteins [4]. The CGE system used in the experiments offers a rapid sugar analysis (<220 sec) for consequent for data analysis. Our future plans include sequential exoglycosidase digestion of the individual sugar structures for further structural elucidation.

Acknowledgement

This work was supported by the MTA-PE Translation Glycomics Grant (#97101) and the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP 4.2.4.A/2-11-1-2012-0001 ‘‘National Excellence Program.’’ Provision of the CGE system by BiOptic, Inc. is greatly appreciated. We also acknowledge the generous support of ProZyme Inc. and the inspiring discussions with Drs. Zoltán Szabó and Ted Haxo.

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P53 SYNTHESIS, ANALYSIS AND BIOLOGICAL EVALUATION OF SOME GLYCOCONJUGATES

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1 Introduction

Glycoconjugates are compounds consisting of carbohydrate moiety attached to an aglycone core. These types of molecules are widely occurring in nature and also often exhibit interesting biological properties [1].

In our ongoing research we focused on the synthesis of a series of glycosides bearing various aglycones attached to either monosaccharide or disaccharide unit. The resulting glycoconjugates were subsequently tested for antimicrobial activity.

2 Experimental

The glycosides derived from monosaccharides such as D-mannose (**1**), D-glucose (**2**), D-galactose (**3**), and disaccharide D-cellobiose (**4**) (Fig. 1) were synthesized over three steps (Fig. 2) in moderate to good yields by the following reaction sequence: acetylation of sugar, glycosylation of an alcohol with the acetylated sugar and Zemplen deprotection. In each step the product structure was proved by various analytical techniques, mainly by NMR spectroscopy and mass spectrometry. The NMR spectroscopy was important tool to confirm stereochemistry at anomeric centre. In case of glucosides, galactosides and cellobiosides their α- and β-anomers are easily distinguished due to significant differences in their $J_{1,2}$ values. NMR spectroscopy is very important also for determination of the anomeric configuration of D-mannosides since $J_{1,2}$ of both α- and β-anomers is around 1Hz. Evidence for α-stereochemistry of the glycosidic linkages in the target mannosides was only obtained on the basis of the $^1J_{C\text{-}1,H\text{-}1}$ heteronucelar coupling constant for the anomeric carbon atoms which is in the range of 168.9 Hz and 170.1 Hz for α-anomer [2].

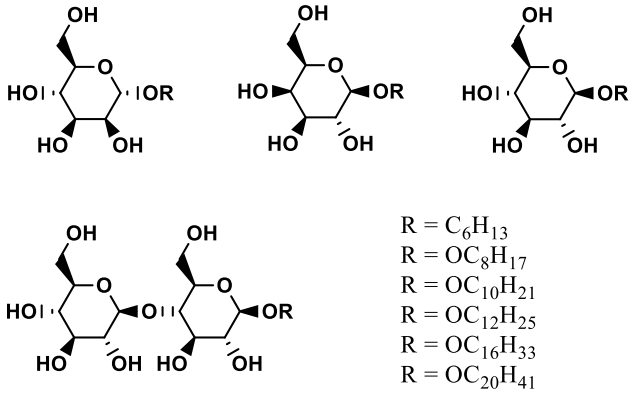

Fig. 1. Test compounds.

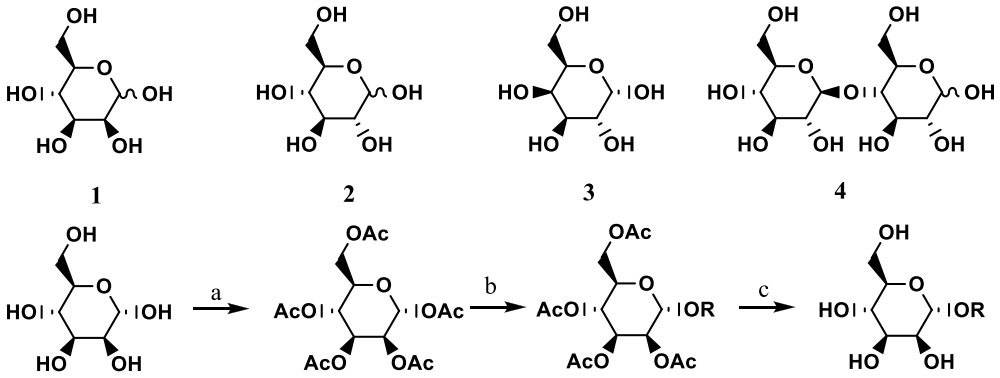

Fig. 2. An example of the reaction sequence.

Reagents and conditions. a) Ac_2O, H_2SO_4 cat.; b) 1 M $SnCl_4$ in CH_2Cl_2, alcohol, CH_2Cl_2, 16 h, rt, or $BF_3.OEt_2$, alcohol, CH_2Cl_2, 16 h, rt; b) MeONa, MeOH/CH_2Cl_2, 16 h, rt.

3 Results and Discussion

3.1 Synthesis

We prepared a series of glycosides that bear a lipid-like aglycone having a chain of six to twenty carbon atoms. All target compounds were fully characterized by NMR spectroscopy, HRMS and optical rotation, lyophilized and stored at -10 °C. They were dissolved immediately before use in the biological tests.

3.2 Antimicrobial activity

Microorganisms were cultivated in the 96-well microtitration plates in the presence of different concentrations of tested compounds in appropriate media. The effect of tested compounds was determined as the lowest concentration which inhibits the growth of microorganisms (MIC).

- MIC values: in the range from mM to μM [3]
- strains tested: *Escherichia coli* CNCTC 377/79, *Staphylococcus aureus* CNCTC 29/58 and *Candida albicans* CCM 8186
- effect of aglycone on the inhibitory activity
- the best MIC values towards *Candida* and *Staphylococcus* strains

An inhibition of the growth of *Candida albicans* laboratory and clinically isolated strains was investigated.
- in experiment with the laboratory strain inhibition zones were visible only with C8-C12 mannosides
- in experiment with the clinical strain the clotrimazole was used as a negative positive control and two of the tested monosaccharide glycosides were better inhibitors than clotrimazole. This observation was also confirmed by XTT test that showed a reduction of metabolic activity for all tested compounds [4].

4 Conclusions

Some of the tested compounds showed promising activity that opens a possibility for further modification of the glycosides with the aim to develop more active carbohydrate-based compounds.

Acknowledgement

This work was supported by the the Slovak Research and Development Agency (Contract No. APVV-0484-12) and the project VEGA-2/0159/12.

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P54 HPLC SEPARATION OF HUMAN HEMOGLOBINS IN BLOOD SAMPLES OF PATIENTS WITH THALASSEMIA

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Summary
This work was primary focused on separation of clinically significant hemoglobins by HPLC. The separation was performed by the ion-exchange columns PolyLC 50mm and 200mm (5μm; 4,6mm;100nm). For the purposes of study the reproducibility of retention times and peak areas were used separation conditions recommended by the manufacturer of the column. For the study were used blood samples of patients with thalassemia and blood samples of healty people. It was observed, that the percentage of normalized peak area of hemoglobins HbA, HbA2 and HbF vary compared to the blood of healthy people.

1 Introduction

Hemoglobin is metalloprotein of red blood cells whose primary function is to transport oxygen from the lungs to the tissues. A molecule of human hemoglobin is composed of four polypeptide chains, each of which contains a prosthetic group-heme. In the prenatal period and in the period of one year life of the infant it creates mainly fetal hemoglobin (HbF), which consists of two α and two γ polypeptide chains and has a higher affinity for oxygen than hemoglobin of adults. After this period, persists HbF proportion to one volume percent in the blood. Hemoglobin of human adult (HbA) consists of two α and two β polypeptide chains. Hemoglobin HbA_2 which consists of two α and two δ polypeptide chains is also a part of the blood of adults in the amount of three volume percent, but its function is yet unknown [1].

Genetically determined disorders of hemoglobin production may cause either insufficient synthesis of α or β globin chains, such is known as thalassemia or mutations in globin genes result in substitutions in the primary structure of globin chains and the formation of abnormal hemoglobins species (eg, hemoglobin S and hemoglobin C etc.) and therefore are referred to as hemoglobinopathies [2].

The correct diagnosis of those rarer types of anemia is particularly important because of the exclusion of inappropriate drug treatment and prenatal prevention. Even though they are already available several commercial HPLC and capillary electrophoresis machines for separation and determination of hemoglobin, the separation of hemoglobins is not optimal.

2 Experimental

2.1 Chemicals

HCl 37% (V/V) and KCN from Merck (Darmstadt, Germany), NaCl and Bis-tris from Sigma-Aldrich (St. Louis, USA) all in analytical grade. $KMnO_4$ from Lachema (Brno, Czech republic). Samples of human blood were provided from St. Michael's Hospital, Bratislava, Slovakia. All aqueous solutions were diluted with ultrapure water purified by Millipore Simplicity (Molsheim, France).

2.2 Chromatography

The HPLC system (Agilent Technologies 1260) consisted of the following devices: mobile phase vacuum degasser (G1379B), dual-channel high-pressure pump (G1312B), auto sampler (G1329B), column thermostat (G1316B), DAD (G1315). Separation was performed on ion-exchange columns PolyLC 50mm and 200mm (5μm particle size; 4.6mm i.d.; 100 nm pore size; PolyLC Inc.; Columbia; USA). On columns were separated blood samples of healty adults and adults with thalassemia and AFSC standard solution (a mixture of HbA, HbF, HbS and HbC) used in electrophoresis.

2.3 Sample preparation

Patient blood was collected into tubes containing potassium chelaton as an anticoagulant. Subsequently, the blood was centrifuged at 13400 rpm during 10 minutes. The precipitate was separated from the liquid supernatant and used for further purification by means of saline solution. Saline solution with red blood cells was shaken and then centrifuged at 13400 rpm during 10 minutes. Purification with saline solution was repeated two times. Then was 100μl of precipitate red blood cells hemolysed by 300μl of water during 15 minutes. Hemolysate was filtered through 0.22 μm microfilter and injected in to the column.

3 Results and Discussion

Blood of adult with thalassemia compared with the blood of healthy people have higher levels of HbA_2 and HbF, while the amount of HbA is lower (see Figure 1). It is because of synthesis β-globin chains in β-thalassemia is insufficient, what results in increased production of hemoglobins without β-globin chains in structure. Rise in HbA_2 and HbF is different in patients. For example, chromatogram b in Figure 1 shows only a proportion of HbA_2 is increased (more than 3 percent by volume), while the proportion of HbF is normal (up to 1 volume percent). On the chromatogram c are amounts of both mentioned hemoglobins elevated above limits.

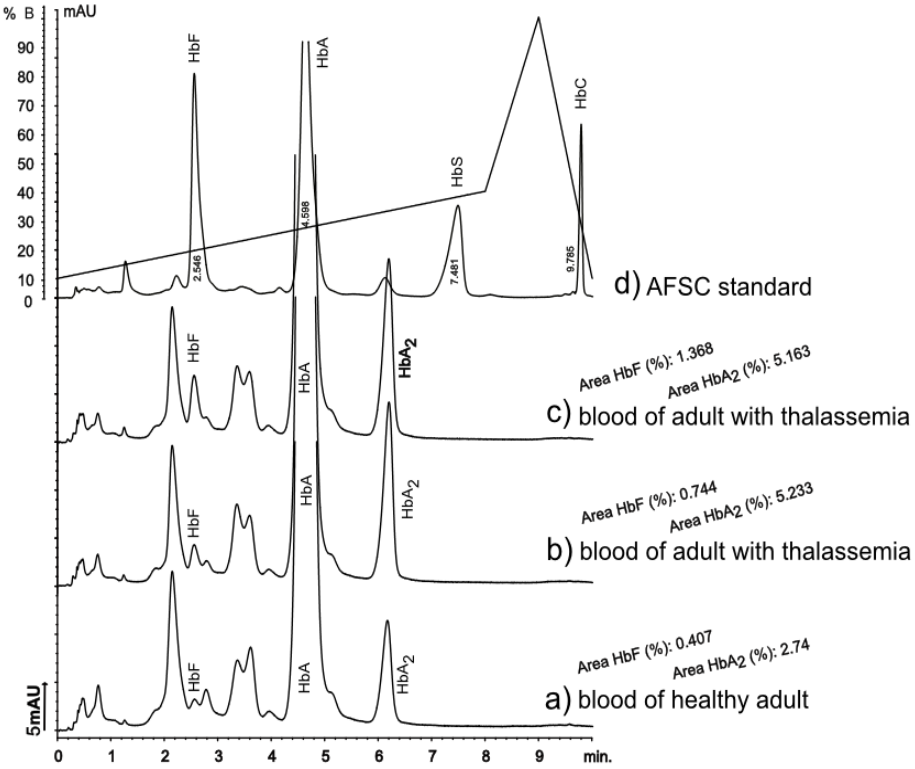

Fig. 1. Chromatographic results from separation of blood samples on 50mm column. Chromatographic column PolyLC 50mm (5µm particle size; 4.6mm i.d.; 100nm pore size), mobile phase A: pH 6.96; 20mM Bis-Tris, 2mM KCN; mobile phase B: pH 6.65; 20mM Bis- Tris, 2mM KCN, 200mM NaCl, gradient at a flow rate 1.7 ml min^{-1}, injected volume 8 µl, UV detection at 210 nm.

By using longer column better resolution was obtained for HbF and HbA from the other components of the sample, but the separation time has almost tripled (see Figure 2).

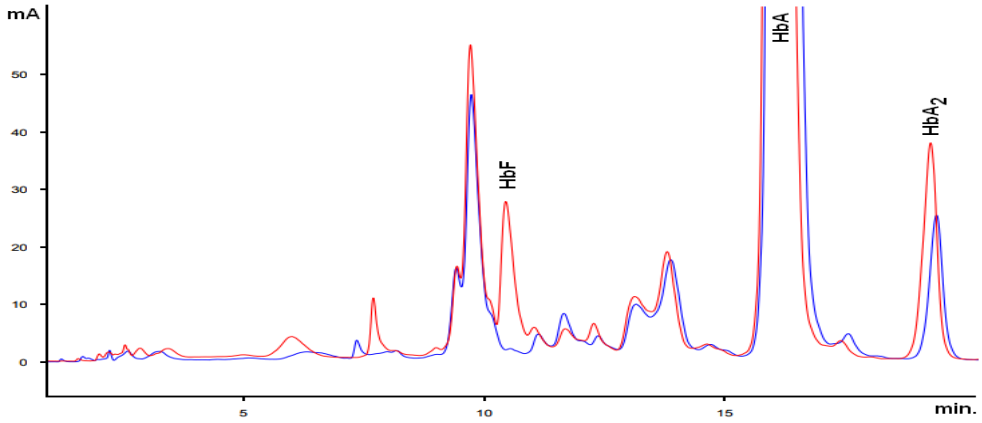

Fig. 2. Chromatographic results from separation of blood samples on 200mm column. Chromatographic conditions as in Fig. 1.

4 Conclusions

From the chromatograms of blood samples of patients with thalassemia could be observed, that the percentage of normalized peak area of hemoglobins HbA, HbA_2 and HbF vary compared to the blood of healthy people. Study the reproducibility of retention times and peak areas was evaluated for HbA_2, because only this one was resolved to baseline. Relative standard deviation of retention times at 23 repetitions was 0.43% and for peak areas it was 7.24%. Reproducibility of peak area decreased with time. After three days of measurements on the column without flushing it with EDTA solution was the symmetry of the peaks, the separation efficiency and thus the reproducibility of measurements adversely affected. Overall, it can be stated that the above HPLC method, which is almost standard used method, is relatively low robust HPLC method and therefore requires further study and improvement.

Acknowledgement

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P55 AFFINITY CAPILLARY ELECTROPHORESIS APPLIED FOR STUDY OF SOLVENT EFFECT ON STABILITY CONSTANT OF DIBENZO-18-CROWN-6 COMPLEX WITH POTASSIUM ION

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Summary

Affinity capillary electrophoresis (ACE) was used for investigation of solvent effect on stability constant of model complex of dibenzo-18-crown-6 with potassium ion in mixed hydro-organic solvents at different water/methanol and water/ethanol ratios. The stability constant of the complex was found to be strongly decreasing with

increasing molar fraction of water in mixed solvents and almost independent of the organic solvent used.

1 Introduction

Together with a stoichiometry, stability (affinity, binding) constants of complexes are their most important characteristics. The stability constants are much more frequently determined in pure solvents than in the mixed solvents [1]. Nevertheless, the knowledge of stability constant dependency on the composition of the mixed solvents might be useful in optimization of some processes, e.g. catalysis or separation. Stability constants can be determined using various techniques including spectrophotometry, mass spectrometry, electrochemistry, calorimetry or electromigration methods [2]. Affinity capillary electrophoresis (ACE) is widely used for stability constant determination because of the small sample and electrolyte consumption, simplicity of the data evaluation and some other advantages [3-6].

The aim of this study was to apply ACE for investigation of influence of composition of mixed hydro-organic water/methanol and water/ethanol solvents on the stability constant of dibenzo-18-crown-6 complex with potassium ion.

2 Experimental

Dibenzo-18-crown-6 (98 %), methanol (HPLC grade), dimethyl sulfoxide (≥ 99.5 %), potassium chloride (analytical grade) and acetic acid (≥ 99.8 %) were purchased from Sigma-Aldrich (Steinheim, Germany), ethanol (absolute) was from Penta (Prague, Czech Republic).

ACE experiments in water/methanol mixtures were performed in Agilent G1600AX CE apparatus and Agilent 7100 CE System (Agilent, Waldbron, Germany) was used for measurements in water/ethanol mixtures. In both analyzers, the internally uncoated fused silica capillary (375/50 μm, OD/ID, 40/29 cm total/effective length) was used and UV-absorption detection at 206 nm was employed. The separation voltage varied from 6 kV to 26 kV. Background electrolyte (BGE) consisted of 25 mM lithium hydroxide and acetic acid in concentration necessary for constant pH 5.5. Portion of methanol (ethanol) in the solvent mixture varied from 0 to 100 % v/v. The pH was calculated using pK_a values of acetic acid in the particular mixed solvents [7, 8] and measured by pH meter CyberScan PC 5500 equipped with glass electrode. Concentration of KCl in BGE varied from 0 to 10 mM (in the case of very weak complexes from 0 to 30 mM). For the data evaluation and stability constant calculation, the programs MS Excel 2010 and Origin 8.5.1 were used.

3 Results and Discussion

Determination of stability constant of ligand-ion complexes by ACE is based on measurement of effective electrophoretic mobility of the ligand in the presence of complexing ion in the BGE. In this study, the stability constant of dibenzo-18-crown-6 (ligand, L) complex with potassium ion (general metal ion, M^+) was determined by ACE in mixed hydro-organic solvents at different water/methanol and water/ethanol ratios. In this method, the stability constant of ML complex (K_{ML}) is obtained by

nonlinear regression analysis of dependency of effective electrophoretic mobility of the ligand ($\mu_{L,eff}$) on the concentration of complexing ion ([M^+]) in the BGE using an equation (1):

$$\mu_{L,eff} = \frac{K_{ML}[M^+]\mu_{ML}}{1+K_{ML}[M^+]} \quad (1)$$

where μ_{ML} is the mobility of the complex ML. If the ligand interacts with some other component of the BGE, the mobility of the ligand in the absence of metal cation (μ_L^*) is taken into account and eq. (1) is modified to eq. (2):

$$\mu_{L,eff} = \frac{\mu_L^*+K_{ML}^*[M^+]\mu_{ML}}{1+K_{ML}^*[M^+]} \quad (2)$$

In this case, the obtained constant is called conditioned stability constant K^*_{ML}.

The measured dependencies fitted with the eq. (1) and eq. (2) are shown on Figure 1.

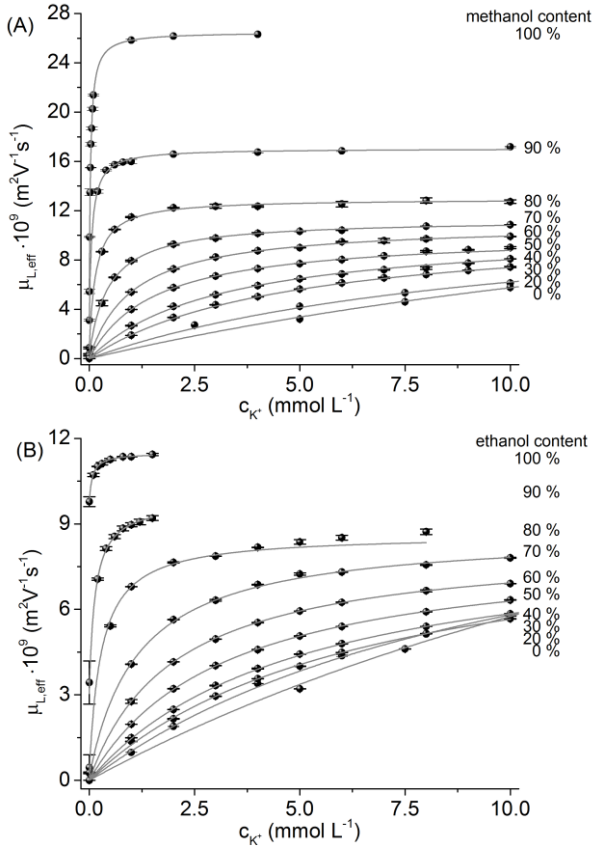

Fig. 1 Dependencies of effective electrophoretic mobility of the ligand, $\mu_{L,eff}$, on concentration of potassium ions in BGE, c_{K+}, at different ratios of methanol/water (A) and ethanol/water (B) mixed solvents.

The aim of this work was to find the solvent properties influencing the stability constant of the complex. Hence, the logarithm of stability constant of the complex was plotted as a function of relative permittivity of the mixed solvents, which represents their relevant physico-chemical quantity. Two close to linear dependencies were obtained; with a steeper decrease for methanol/water mixtures

than for ethanol/water mixtures, see Fig. 2A. In addition, the relation between stability constant of the complex and molar fraction of water in the mixed water/methanol and water/ethanol solvents was studied as well.

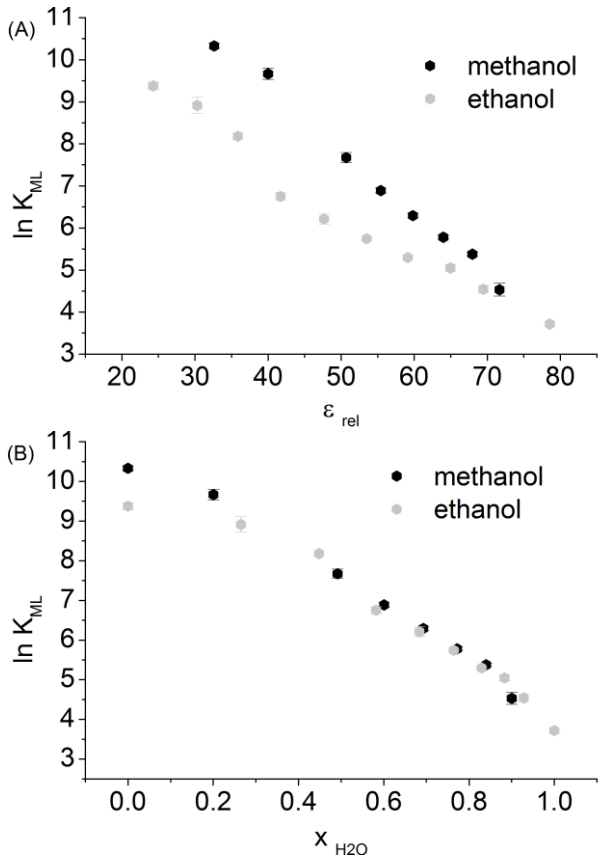

Fig. 2. Dependencies of logarithm of stability constant K_{ML} on (A) relative permittivity of the water/methanol and water/ethanol mixed solvents, and on (B) molar fraction of water in these mixed solvents.

Figure 2B shows that the logarithm of stability constant of the complex is strongly dependent on the molar fraction of water in the solvent mixture rather than on the sort of the organic solvent used. This dependency confirms assumption that the water molecules present in the solvent mixture surround the central metal ion and shield it from the ligand.

4 Conclusions

ACE study of dibenzo-18-crown-6 complex with potassium ions in mixed hydro-organic solvents at different water/methanol and water/ethanol ratios has shown that the stability constant of this complex is strongly decreasing with increasing relative permittivity of the mixed solvents as well as with increasing molar fraction of water in these mixed solvents. On the other hand, the stability constant is almost independent of the organic solvent used. It confirms the assumption of shielding the central metal ion by water molecules.

Acknowledgement
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P56 ELECTROPHORETIC ANALYSIS OF SERUM PARAPROTEIN GLYCOSYATION IN MULTIPLE MYELOMA

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Summary
Multiple myeloma (MM) is a currently incurable malignancy of human plasma cells. Its pathogenesis is poorly understood, and mounting evidence indicates that the bone marrow microenvironment of tumor cells has an eminent role in the process. This work reports on a novel approach for paraprotein glycosylation analysis from intact and papain digested forms. Capillary Electrophoresis with Laser Induced Fluorescence detection (CE-LIF) was used for the analysis of the glycosylation patterns.

1 Introduction

Multiple myeloma (MM) is characterized by local proliferation of malignant plasma cells. Recently, several new and unexpected oncogenic mechanisms were suggested by the pattern of somatic mutation on the basis of data. These include the mutation of genes involved in the protein translation (paraproteins), histone methylation, blood coagulation and post translational modifications, such as IgG glycosylation. In addition, the role of NF-κB pathway is important as well as there is potential immediate clinical relevance activating mutations of the kinase BRAF in MM development [1]. It has been shown that in multiple myeloma (MM) each IgG paraprotein exhibits a unique oligosaccharide profile [2]. The conventional treatment of the disease is chemotherapy [3], however, several new prototypic drugs (thalidomide, lenalidomide, bortezomib) were recently introduced for treatment benefiting multiple myeloma and other haematological malignancies tumor patients. The durability of response to novel drugs in newly diagnosed patients and the benefit of high-dose melphalanand stem-cell transplantation in young patient so increasing chance of survival. Early diagnosis of disease is important to define adequate treatment and consequently for long time survival. IgG free light chains (FLCs) are traceable in patients with multiple myeloma by immunoassays, which provide quantitative measurement of FLCs in serum and MM patients having abnormal serum FLC ratios [4]. Patients with MM in most instances show abnormal immunoglobulin patterns (paraproteins) exhibiting unique N-linked oligosaccharide profiles. It has been suggested that this is a result of changes in clone specific glycosylation machinery. The data presented here support that physiological environments in multiple myeloma may profoundly influence paraprotein glycosylation [2], thus its analysis is important for diagnostic purposes to reveal disease specific markers for MM.

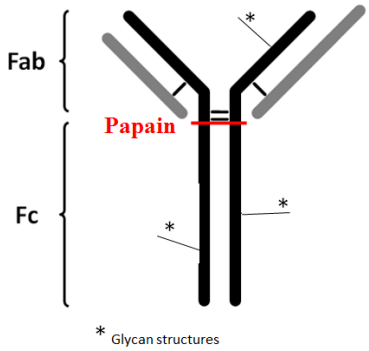

Fig. 1. Papain digestion of IgG. (*) possible glycosylation sites.

2 Experimental

To model paraproteins, standard IgG (Sigma-Aldrich, St. Luis, MO) was papain digested (Sigma-Aldrich) and purified with Protein A columns (PhyNexux, San Jose, CA). Cleavage effectivity and binding of the F_c portion was analyzed with SDS-PAGE after elution. The flow through containing the Fab fragments were further purified by Fabκ and Fabλ bead containing pipette tips (PhyNexux, San Jose, CA) and

analyzed with SDS-PAGE using a Mini-PROTEAN System (Bio-Raad, Hercules, CA). The separation gel (12,5%) contained 2.5 ml Protogel, 1,5 ml Protogel Gel buffer, 2,0 ml H_2O, 60 µl SDS (10%), 60 µl APS (10%), 6 µl TEMED) and the stacking gel contained 0.333 ml Protogel, 0,625 ml stacking buffer, 1,525 ml H_2O, 25 µl SDS (10%), 25 µl APS (10%), 2,5 µl TEMED). 20 µl denatured samples and marker ladder (Sigma marker wide range, Sigma-Aldrich Company Kft. Hungary,) have been injected and then 120V electric field was applied for 2 hours. After the run, the gels were stained by Coomassie Brilliant Blue for 20 min and destained in 50% methanol, 7% concentrated acetic acid and 43% water and overnight.

Partitioned IgGs and their fragments (Fc and Fab) were digested with PNGase F enzyme for the removal of their carbohydrate moieties. The released glycans were dried in centrifugal vacuum evaporator. The liberated glycans, the maltodextrin ladder and maltose internal standard were all fluorophore labeled by 6 µl aminopyrenetrisulfonic acid (APTS, Beckman Coulter, Brea, CA) and 2 µl of 1 M sodium cyanoborohydride in tetrahydrofuran at 37°C overnight. The unreacted fluorescent dye was removed by cleanup cartridges (ProZyme, Hayward, CA). Briefly, the binding step was performed in 200 µL 1x cleanup solution to each glycan sample, following by washing off the unreacted dye from the cartridges with 200 µL 1x cleanup solution, and finally the elution of glycans by water.

Capillary electrophoresis profiling of the APTS-labeled N-Glycans was performed in a P/ACE MDQ automated CE instrument (Beckman Coulter) equipped with a fluorescent detector using an Ar-ion laser (excitation 488nm, emission 520 nm). The separations were accomplished in 50 cm effective length (60 cm total) neutral coated 50 µm i.d. capillary columns filled with the N-CHO Carbohydrate Separation Gel Buffer (Beckman Coulter). The applied electric field strength was 500 V/cm.

3 Results and Discussion

The flowchart of Figure 2 shows the individual steps followed to compare the normal and MM patient plasma samples. SDS-PAGE analysis (Figure 3) revealed different IgG light chain migration of control and patient samples (lanes A and B-P) and the analysis of papain digested samples showed visible differences as well (lanes C and D).

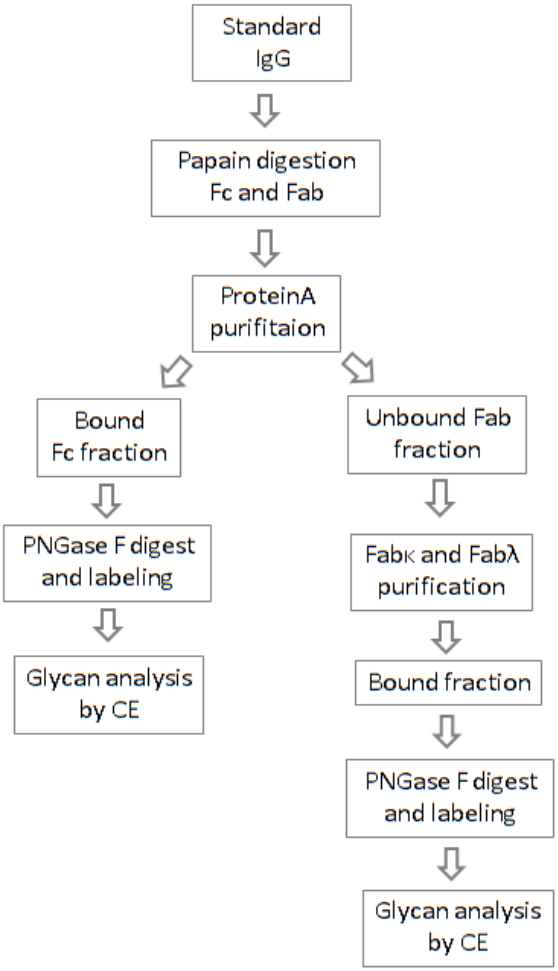

Fig. 2. IgG glycosylation pattern analysis workflow.

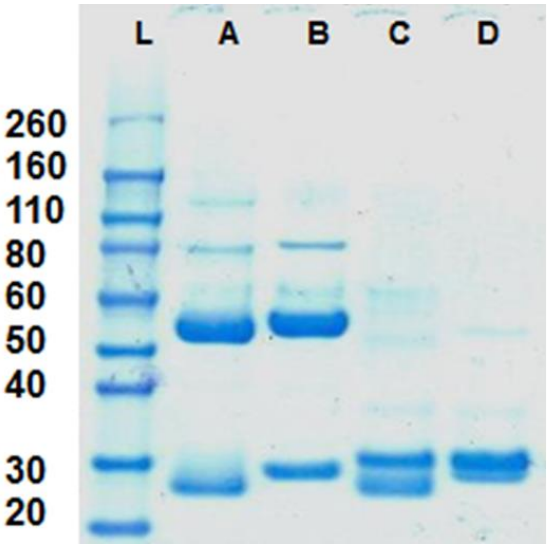

Fig. 3. SDS PAGE of the Protein A bound fraction of control and MM patient samples, following the workflow delineated in Figure 2. Lanes: L: protein ladder, A: normal patient sample (Protein A purified), B: MM patient sample (Protein A purified), C: papain digested sample A, D: papain digested sample B.

Analysis of Fc and Fab regions and their endoglycosidase digestion of the MM patient sample suggested that glycosylation was present on both the Fc and Fab parts of the heavy chains as well as on the light chains. PNGase F digested normal and MM patient samples were analyzed in CE-LIF system and different electropherograms were showed this experiment.

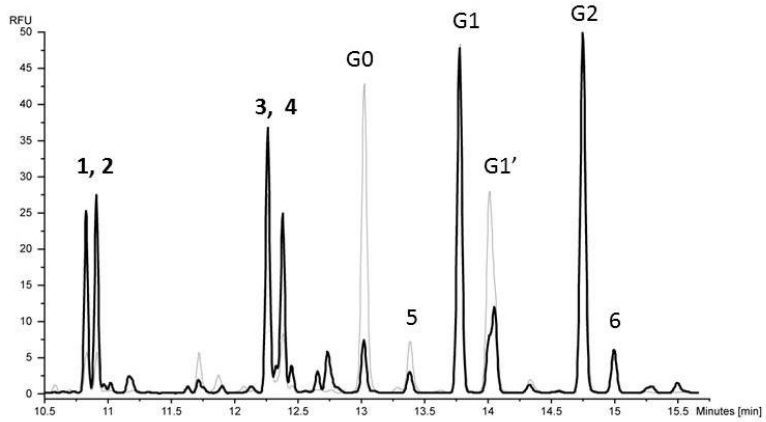

Fig. 4. Total glycan profiles of the Protein A partitioned normal and MM patient plasma samples.

Figure 4 shows the electropherogram of total Protein A partitioned plasma glycans of the normal (gray) and MM patient (black). It is clearly observable that in case of the normal patient sample, only the regularly expected IgG glycans are present (Peaks G0, G1, G1' and G2). On the other hand, in case of the MM patient sample, highly (Peaks 1 and 2) and moderately (Peak 3) sialyated glycans are visible in addition to the regular IgG glycans of G0, G1, G1' and G2. The additional peaks of 5 and 6 are also characteristic of the unconventional paraprotein glycosylation.

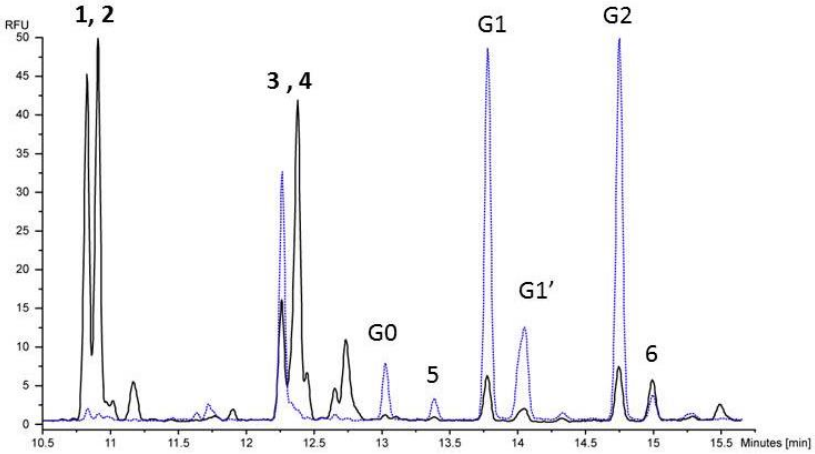

Fig. 5. Glycosylation analysis of the Fc (blue) and Fab (black) portions of the MM patient.

Figure 5 depicts the N-glycan analysis of the papain digested Protein A partitioned fraction of the MM patient. The blue trace represents the glycan pattern of

the Fc portion, while the black trace represents the glycans released form the similarly treated Fab fraction. In this instance it is clearly visible that the highly sialylated glycans originated from the Fab fraction while the moderately sialylated ones were present on both fragments. The larger glycan structures migrating after the G2 peak are specific for the Fc fraction.

4 Conclusions

This paper reports on glycosylation pattern characterization of normal and MM patient plasma samples. Comparison of the standard total Protein A partitioned glycans with the papain digested fragments (Fc and Fab) revealed subtle differences. Based on these preliminary results, the next step will be full structural analysis of these glycans and validation of these potential glycan biomarkers in a larger cohort.

Acknowledgement

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P57 OPTIMISATION OF SAMPLE PREPARATION FOR THE DETERMINATION OF LIPOIC ACID IN VEGETABLES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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Summary

The method developed for determination of lipoic acid in food samples is based on conversion of lipoic acid to dihydrolipoic acid. The first step includes reduction of disulfide bond of lipoic acid with tris(2-carboxyethyl)phosphine. During this reaction disulfides are converted into their thiol derivatives with –SH function. Next step contains precolumn derivatization of dihydrolipoic acid with 1-benzyl-2-

chloropyridinium bromide to form stable 2-S-pyridinium derivative. For determination of derivative ion-pairing reverse-phase liquid chromatography with ultraviolet detection at 321 nm was used. In developing this method the following parameters were investigated and optimized: stability of lipoic acid in different solvents, volume of solvent and time of extraction, drying temperature of extract. The calibration curve was linear in the test ranges 0.2-1 and 1-20 nmol/g potato. The method was validated for potato samples spiked with standard solution of lipoic acid.

1 Introduction

Lipoic acid (LA, 6,8-thioctic acid, 1,2-dithiolane-3-pentanoic acid) is a natural antioxidant present in prokaryotic and eukaryotic cells. This compound exist in oxidized and reduced forms [1, 2, 3]. LA is an essential cofactor for mitochondrial enzymes and a natural antioxidant as it can scavenge reactive oxygen species. This compound inhibit lipid peroxidation products, and ameliorate oxidative damage [2]. α-Lipoic acid supplementation is recommended in the treatment of neurodegenerative disorder, heavy metal poisoning, AIDS, diabetes, radiation damage, liver diseases (mushroom poisoning or alcoholic liver disease) [1, 3]. α-Lipoic acid, which can increase the levels of sulfane sulphur and hydrogen sulfide, protects the heart against myocardial ischemia-reperfusion injury [4].

Therefore, the determination of LA in biological samples is important in biochemistry, nutritional and clinical chemistry study. Several techniques, such as gas chromatography, high-performance liquid chromatography (HPLC), and capillary electrophoresis, have been used for the separation of LA in various samples [2, 3].

2 Experimental

2.1 Chemicals and reagents

As a reducing agent of disulfide bonds tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was used. As a derivatization reagent was used 1-benzyl-2-chloropyridinium bromide (BCPB) which was synthesized in our laboratory [5]. In our studies other reagents were used: lipoic acid, acetonitrile, acetic acid, perchloric acid, tris(hydroxymethyl)aminomethane, hydrochloric acid, methanol, dichloromethane, ethyl acetate.

A primary stock solutions were prepared by dissolving appropriate amount of α-LA (final concentration 0.1 mol/L) in 0.1 mol/L NaOH, BCPB (0.1 mol/L) in water and TCEP (0.25 mol/L) in TRIS buffer (pH=9). The working solutions were prepared by serial dilution with water.

2.2 Sample preparation

Vegetables, which were used in the study were purchased at the local market. Fresh potatoes were peeled, cut in cubes and 1 g of the edible part were crushed and homogenized. Food samples with spiked standard solution of LA were extracted with 3, 5, 7, 10 mL of ethyl acetate for 5, 10, 15, 20 and 30 min. Then 500 µL of supernatants of the extracts were evaporated to dryness in 75, 80 and 100 °C. The

residues were dissolved in TRIS buffer (pH=9) and reduction reaction and derivatization were performed.

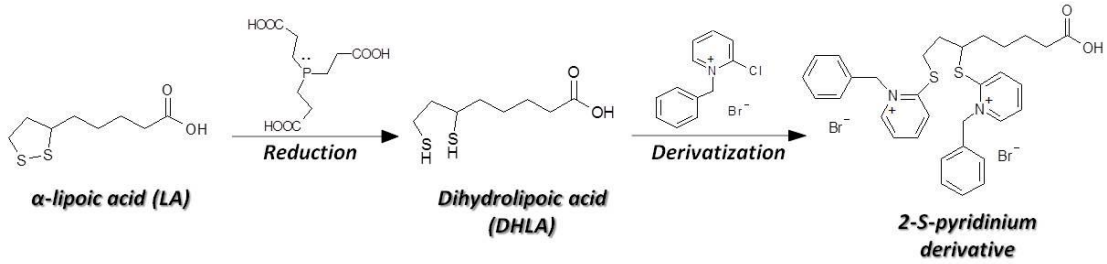

Fig.1. Scheme of reduction of LA and conversion of DHLA to form 2-S-pyridinium derivative.

Stability study: standard solution of LA in different solvents (methanol, dichloromethane, acetonitrile, ethyl acetate) was prepared in the morning and 50 µL of sample was collected at 0 min, 30 min, 2 h, 3 h, 5 h, 24 h and processed for the determination of LA.

2.3 Instrumentation and chromatographic conditions

The chromatographic analysis were performed using an Agilent Technologies 1220 Infinity LC system equipped with diode-array detector. All separations were performed with the use of the Poroshell 120 SB-C18 analytical column (100 × 4.6 mm, 2.7 µm, Agilent Technologies). Chromatographic separations were achieved isocratically using mobile phase consisted of 2% acetic acid solution, pH=2.46 (75%) and acetonitrile (25%) at a flow rate of 1.0 mL/min and the peaks were monitored at 321 nm. Total run time of one analysis was 5.50 min.

3 Results and discussion

3.1 Stability of LA in different solvents

Stability of standard solution of LA in different solvents (methanol, dichloromethane, acetonitrile, ethyl acetate) was tested. It was observed that the solution of LA in methanol, acetonitrile and ethyl acetate is stable for 24 hours while LA in dichloromethane was unstable.

3.2 Extraction of LA

In addition, volume, time and different extraction solvents (methanol, dichloromethane, acetonitrile and ethyl acetate), and temperatures of drying were tested. Among the various conditions studied, the best results were obtained for 30 min extraction of the sample (1 g) with 5 mL of ethyl acetate. The extraction yield was 84.3%. Optimal temperature to evaporate extract to dryness was 80 °C. Higher temperatures cause the loss of LA in tested samples.

3.3 Validation study

The relationship between LA concentration and peak height of LA-BCPB derivative was found to be linear in tested ranges 0.2-1 and 1-20 nmol/g potato. The equations for the linear regression lines were y=1.7382x-0.2453 (R^2=0.9981) and y=0.7876x+08867 (R^2=0.9996) for ranges 0.2-1 and 1-20 nmol/g, respectively. The relative standard deviation values were within 1.1-15.7% (n=3). The recovery of the procedure varied from 77.9% to 109.4%.

4 Conclusions

The report describes first part of research concerning determination of lipoic acid in food samples. The method is based on several steps: sample extraction with ethyl acetate, reduction and derivatization reactions. Developed method is simple, cheap and can be applied to determine LA content in food matrices.

Acknowledgement

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P58 ANALYSIS OF POLYPHENOLS IN SELECTED BLACK AND GREEN TEAS BY HPLC-MS TECHNIQUES

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1 Introduction

The phenolic content in tea refers to the phenols and polyphenols, natural plant compounds, which are present in teas. These chemical compounds affect the organoleptic characteristics of food and beverages [1]. The polyphenols or catechins

in green and black teas are known to posses many benefits on human health. These species have antioxidative activity and also play an important role in prevention of cardiovascular diseases and osteoporosis. The antioxidative activity of catechins help improve hypertension, reduce inflammation and improve cognitive dysfunction [2]. Polyphenols in tea include catechins, theaflavins, tannins and flavonoids [1]. The content of individual phenols in tea varieties is highly variable and depends on fermentation procedure and geographical region. According to the degree of fermentation, the teas are distinguished into three groups: non-fermented (green, white and pu-erh teas), partially fermented (paochong and oolong teas), and fully fermented (black and pu-erh teas) [3, 4]. The tea infusion brewed from Camellia sinensis (L.) is the most popular consumed beverage in the world after the water [5].
In this study, a simple, rapid and efficient high performance liquid chromatography (HPLC) method combined with mass spectrometric (MS) detection was used for characterization and identification of several phenolic compounds present in selected tea varieties (black teas and green teas) from different producers.

2 Experimental

2.1 HPLC-MS analysis

HPLC-MS analyses of selected black and green tea samples and their phenolic profiles were performed by LCMS-IT-TOF^{TM} analyzer (Shimadzu, Kyoto, Japan) equipped with electrospray ionization (ESI) operating in positive and negative ionization mode. HPLC separations were performed on Kinetex XB-C18 column (100 x 2.1 mm; 2.6 m) (Phenomenex, Torrance, CA, USA) using gradient elution: water + 0.1% formic acid (A)– acetonitrile + 0.1% formic acid (B) with 0.2 ml/min flow rate (0 min.: 5% B; 2 min.: 10% B; 6 min.: 40% B; 7 min.: 70% B; 8 min.: 70% B; 8.1 min.: 5% B; 8.2-15 min.: 5% B. The column was thermostated to 40°C. Data in the MS experiments was acquired automatically within 50-1000 m/z values in both the positive and negative modes and within 190-400 nm wavelengths during DAD detection. Data acquisition and data evaluation were performed by using LCMS Solution ver. 3.51 (Shimadzu). MSXelerator software ver. 2.4 (MSMetrix, Maarssen, Nederland) was used for classification of individual tea samples and their phenolic profiles. Total analysis time was 15 minutes and injected volume was 2 µl.

2.2 Chemicals

Chemical used in this work were obtained from Merck (Merck, Darmstadt, Germany) and Sigma-Aldrich (Sigma-Aldrich,Steinheim, Germany).

Samples

Six samples of teas from different producers were purchased in local supermarket (see Table 1**).** The tea extracts were prepared as follows: individual tea sample (1.5 g) was extracted with 150 ml of boiling water. Extraction time was 5 minutes. The tea extracts were cooled to laboratory temperature (23.5 °C) before sampling.

Table 1. Selected tea samples analyzed in this work.

PRODUCT	PRODUCER
Green Tea: Orange and Lotus flower	MISTRAL
Green Tea: Acerola cherry & Elderflower	PICKWICK
Green Tea: Intense mint	LIPTON
Black Tea: Earl Grey Bergamot	EARL GREY
Black Tea: Darjeeling	LORD NELSON
Black Tea: Golden Sikkim	LORD NELSON

3 Results and Discussion

The first part of this work was to find the optimal conditions for HPLC separation of phenolic compounds in selected tea samples. Consequently, the mass-spectrometric detection of selected tea samples (black teas and green teas) was performed. Total analysis time was 15 minutes. The individual tea samples, prepared according to the procedure described in part Samples, were injected (2 µl) into the LC-MS ESI-IT-TOF analyzer. MSXelerator software version 2.4 was used for the visualization of obtained data. Obtained chromatograms and MS spectra show the different composition of individual tea samples and their different phenolic profiles (Fig. 1, Fig. 2). It can be caused by using of different fermentation procedure, different geographical region and growing season.

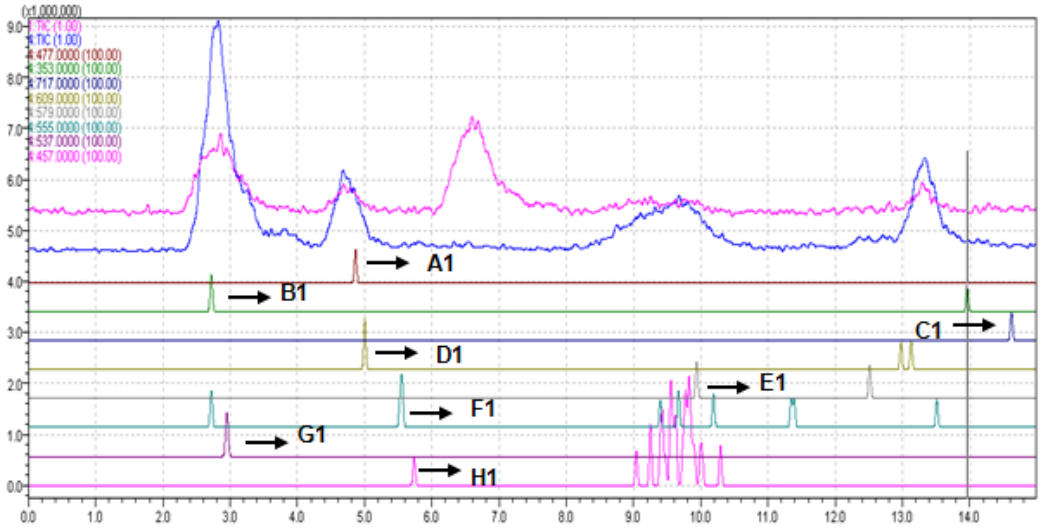

Fig. 1. TIC and EIC traces obtained from HPLC-MS analysis of 2 µl of green tea extract (Acerola cherry & Elderflower- Pickwick) and identified polyphenols: A1- hydroxy luteolin glucuronide, B1- chlorogenic acid, C1- salvianolic acid B derivate, D1- rutin, E1- narirutin, F1- salvianolic acid K derivate, G1- lithospermic acid A isomer, H1- epigallocatechin gallate.

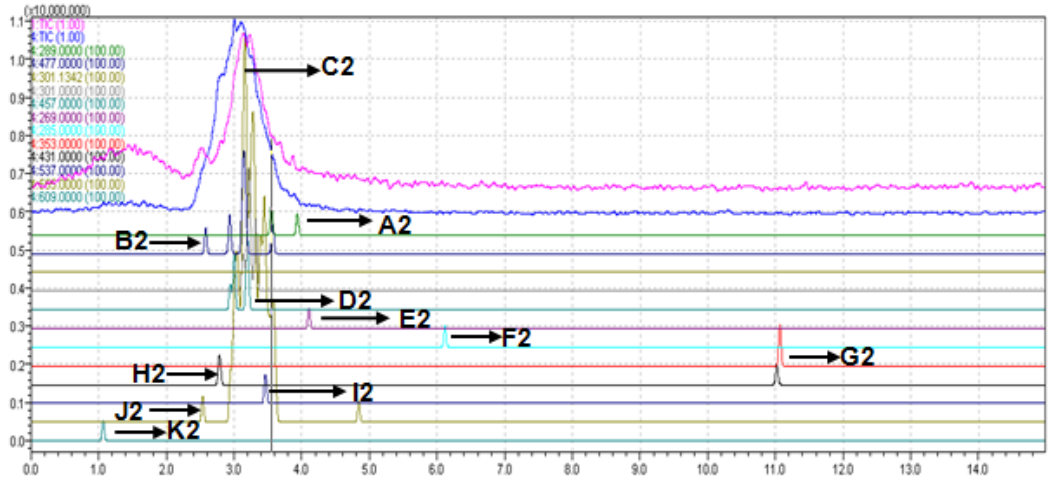

Fig. 2. TIC and EIC traces obtained from HPLC-MS analysis of 2 µl of black tea extract (Darjeeling -Lord Nelson) and identified polyphenols: A2- catechin, B2- hydroxy luteolin glucuronide, C2- quercetin, D2- epigallocatechin gallate, E2- apigenin, F2- luteolin, G2- chlorogenic acid, H2- apigenin-7-O-glucoside, I2- lithospermic acid A isomer, J2- salvianolic acid K derivate, K2- rutin).

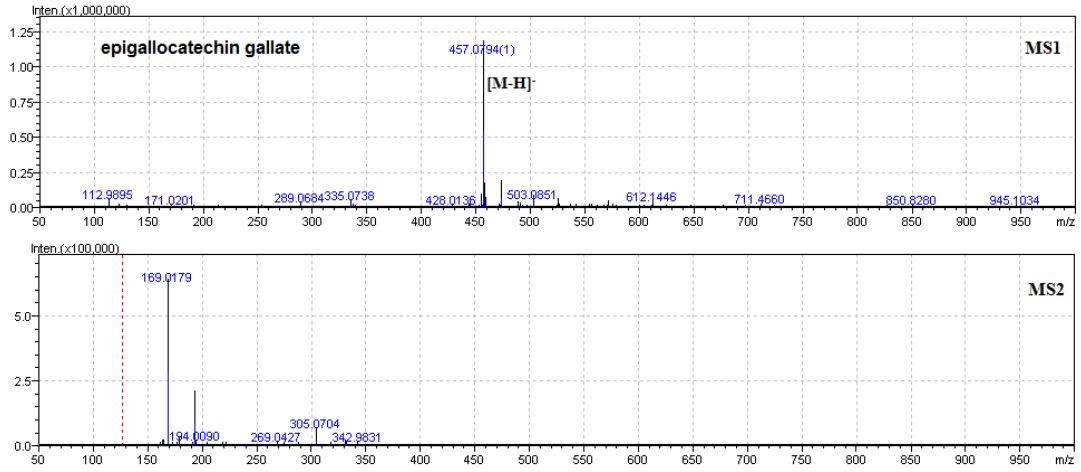

Fig. 3. MS-MS2 spectra of identified phenolic compound- epigallocatechin gallate.

4 Conclusions

The aim of this work was the development of the suitable method based on the combination high performance liquid chromatography and mass spectrometry and its utilizing for the characterization and identification of several phenolic compounds present in selected tea varieties (black teas and green teas) from different producers.

Acknowledgement

This work was financially supported by grants Slovak Research and Development Agency (APVV-0583-11 and APVV-0365-12).

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P59 MICROBICIDAL PROPERTIES AND CHEMICAL COMPOSITION OF ESSENTIAL OILS

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Summary
The microbicidal properties of 6 essential oils (EOs; *Lavandula species*, *Cymbopogon nardus*, *Citrus aurantifolia*, *Juniperus communis*, *Myrtus communis* and *Cinnamomum zeylanicum*) for 17 microorganisms were determined using the vapour-agar contact method. The most effective EO (i.e. *Lavandula angustifolia*) whose volatile components provided the sufficient microbicidal properties was chosen for detailed study of chemical composition. GC-MS was used for the determination of chemical composition of *Lavandula angustifolia* both in the liquid phase and in the gas phase. The components in the gas phase were sampled using a cylindrical wet effluent diffusion denuder into a thin film of n-heptane (used as an absorption liquid).

1 Introduction
Essential oils (EOs) are volatile, natural compounds characterized by a strong aroma. They are formed by plants [1] and isolated from plants mostly by hydro- or steam distillation, by pressing [2] or by fermentation [3]. The EOs are used for example in food and beverage industry or as fragrances in perfumes and cosmetics. In nature, EOs play an important role in the protection of plants as antibacterial, antiviral and antifungal agents [1].

2 Experimental
The microbicidal activity was determined by the modified vapour-agar contact method for bacterial suspension (*Kocuria rhizophila*, *Micrococcus luteus*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Seratia marcescens*), suspension of microscopic filamentous fungi (*Alternaria tenuissima*, *Aspergillus brasiliensis*, *Chaetomium*

globosum, *Cladosporium cladosporioides* f.sp. *pisicola*, *Fusarium oxysporum* f.sp. *lycopersici*, *Mucor racemosus*, *Penicillium aurantiogriseum* var *aurantiogriseum*, *Rhizopus oryzae*, *Serpula lacrymans*, *Trichoderma reesei*, *Ulocladium cucurbitae*) and suspension of bacteria endospores (*Bacillus subtilis*). These experiments were proceeded in desiccators. Glass carriers (a diameter of 2.5 cm) inoculated with examined microorganisms were inserted into the desiccators, together with two Petri dishes. The first Petri dish contained 5 mL of tested EO (*Lavandula angustifolia*, *Cymbopogon nardus*, *Citrus aurantifolia*, *Juniperus communis*, *Myrtus communis* and *Cinnamomum zeylanicum*), and the second Petri dish contained distilled water with NaCl to maintain stable humidity (75 %). The microbicidal efficiency of EOs was evaluated after 1 day, 2 days, 3 days, 1 week, 2 weeks (fungi and bacteria) and also after three weeks (bacterial endospores) of the time when microorganisms were contacted with vapours of the tested EOs for the first time.

The determination of volatile components in the gas phase in desiccators were performed under the same experimental conditions as for the microbicidal activity study. The occurred vapours of components were sampled by using a cylindrical wet effluent diffusion denuder when the components of EOs in gas phase were sorbed into a thin film of n-heptane (used as an absorption liquid) and then the samples were analysed by GC-MS. The amount of components of EOs was expressed as weight percent (wt. %) and the concentrations of volatile components were expressed as ppm v/v (parts per million, volume/volume).

3 Results and Discussion

The saturated vapours of volatile components of *Lavandula angustifolia* completely inhibited tested bacteria and fungi within 3 days and 1 week, respectively. This EO showed the most effective microbicidal properties from studied EOs. *Citrus aurantifolia* was the only one of EOs whose volatiles demonstrated the sporicidal effect against *Bacillus subtilis*. High microbicidal properties of volatile components against bacteria *Proteus vulgaris* and *Seratia marcescens* also showed saturated vapours of *Cinnamomum zeylanicum*. The volatile components of *Cinnamomum zeylanicum* manifested very good antifungal properties against *Aspergillus brasiliensis*. The volatile components of *Cymbopogon nardus*, *Citrus aurantifolia*, *Juniperus communis*, *Myrtus communis* and *Cinnamomum zeylanicum* were also sufficiently effective.

We identified 96.4, 76.2, 97.4, 96.0, 97.7, and 91.5 wt. % of the liquid phase of *Lavandula angustifolia* (Table 1), *Cymbopogon nardus*, *Citrus aurantifolia*, *Juniperus communis*, *Myrtus communis* and *Cinnamomum zeylanicum*, respectively. All these 6 EOs contained 1 – 3 main components that presented 67 – 91 wt. % of total mass of a corresponding essential oil. The amount of other components was much lower (less than 5 wt. %). Eucalyptol and α-pinene were found as the main components of *Myrtus communis* (35.7 wt. % and 35.3 wt. %, respectively). α-Pinene was identified as the primary component of *Juniperus communis* (54.1 wt. %). Limonene (51.4 wt. %) and citronellal (40.4 wt. %) were the main components of *Citrus aurantifolia* and *Cymbopogon nardus*, respectively. Cinnamaldehyde was the

principal component of *Cinnamomum zeylanicum* (62.0 wt. %) and linalool of *Lavandula angustifolia* (51.4 wt. %).

Table 1. Chemical composition of *Lavandula angustifolia* oil and the concentration of its components in the gas phase inside a desiccator within an antimicrobial activity assay.

Component	Weight percent (wt. %) in the liquid phase	Concentration of corresponding components in the gas phase (ppm (v/v))
Tricyclene	Nd (< 0.02)	1.89 ± 0.28
α-Pinene	0.25 ± 0.01	11.3 ± 1.65
Camphene	0.16 ± 0.01	7.82 ± 1.29
β-Pinene	0.21 ± 0.01	9.47 ± 1.20
Myrcene	1.60 ± 0.08	22.2 ± 3.06
3-Carene	0.08 ± 0.01	2.89 ± 0.46
p-Cymene	Nd (< 0.02)	2.12 ± 0.35
Limonene	0.69 ± 0.03	14.9 ± 2.08
Eucalyptol	2.88 ± 0.14	44.9 ± 3.21
β-trans-Ocimene	1.34 ± 0.05	19.7 ± 1.23
β-cis-Ocimene	0.36 ± 0.02	6.33 ± 0.08
γ-Terpinene	0.05 ± 0.01	0.80 ± 0.12
Terpinolene	0.17 ± 0.01	2.44 ± 0.32
Linalool	51.4 ± 2.57	99.0 ± 6.98
Camphor	4.17 ± 0.21	16.7 ± 2.61
Borneol	2.11 ± 0.11	1.88 ± 0.30
Terpinen-4-ol	1.56 ± 0.08	1.21 ± 0.18
α-Terpineol	1.52 ± 0.08	1.02 ± 0.16
Linalyl acetate	26.6 ± 1.33	25.9 ± 3.62
Geranyl acetate	0.48 ± 0.02	0.45 ± 0.06
Caryophyllene	0.76 ± 0.04	0.58 ± 0.09

4 Conclusions

The volatile components of *Lavandula angustifolia* showed the most effective microbicidal properties because they completely inhibited the tested bacteria and fungi within 3 days and 1 week, respectively. The components of *Lavandula angustifolia* with the highest concentration in the gas phase were the following: linalool, eucalyptol, linalyl acetate, myrcene, β-trans-ocimene, camphor and limonene.

Acknowledgement
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P60 IDENTIFICATION OF NOSOCOMIAL INFECTION BY ELECTROPHORETIC TECHNIQUES

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Summary
The detection and identification of pathogens currently relies upon a very diverse range of techniques and skills, from traditional culturing and taxonomic procedures to modern molecular biology based methods. However, conventional laboratory methods are time consuming, laborious, and they may provide both false positive or negative results, especially for closely related microorganisms. In this study, we suggest capillary electrophoresis techniques for differentiation and characterization of Methicillin-resistant and Methicillin-susceptible *Staphylococcus aureus*.

1 Introduction
Treatment of nosocomial infections has become more difficult and more expensive due to the increasing prevalence of multiresistant strains, especially Methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA affects morbidity and costs of treatment of patients compared with infections caused by Methicillin-susceptible *S. aureus* (MSSA) [1]. Rapid detection of low number of *S. aureus* cells (10^1 – 10^2 cells mL^{-1}) in blood is necessary to detect bloodstream infection. A fast detection and identification of MRSA and MSSA also enables to select an appropriate therapy.

2 Experimental
2.1 Bacterial Strains and Growth Conditions
The bacterial strains included in this study, Methicillin-Resistant *Staphylococcus aureus* (MRSA) and Methicilin-Sensitive *Staphylococcus aureus* (MSSA), were obtained from the Czech Collection of Microorganisms and St. Anna University

Hospital (Brno, Czech Republic). The tested strains were cultivated on Muller-Hinton agar at 37 °C for 24 h. Suspension was prepared by re-suspension of microbial cultures in physiological saline solution (PSS). The concentration of the resuspended cells was estimated by the measurement of the optical density of the suspension by means of spectrophotometer at 550 nm, according to the calibration curve, which was defined by reference samples. The numbers of microorganisms in the reference samples were controlled by serial dilution and plating of 100 µL of the suspension on Muller-Hinton agar. After the cultivation at 37 °C for 24 hours, the colonies were counted.

2.2 Equipment and Procedures (CIEF, CZE)

CZE was carried out using a laboratory-made apparatus [2] at a constant voltage (-20 kV on the detector side) supplied by a Spellman CZE 1000 R high-voltage unit (Plainview, NY, USA). The lengths of the FS capillaries, 100 µm I.D. and 360 µm O.D. (Agilent Technologies, Santa Clara, CA), were 35 cm, 20 cm to the detector. Both original and SCW-etched FS capillaries were modified with GOTMS. The ends of the capillary and the electrodes were placed in 3-mL glass vials filled with an anolyte or a catholyte or a background electrolyte (BGE). An LCD 2082 on-column UV-Vis detector (Ecom, Prague, Czech Republic), connected to the detection cell by optical fibers (Polymicro Technologies, Phoenix, AZ, USA), was operated at 280 nm. For CZE separations, 2×10^{-2} mol L^{-1} phosphate buffers pH 5 with addition of 5 % (v/v) EtOH and 0.1 % (w/v) PEG 10 000 were used as BGE. The injection time was 8 s for siphoning action which represents 100 nL of the microbial suspension injected into the capillary. The injection time for the syringe pump was 290 s. The sample segment was composed of 200 nL of BGE, 100 nL of the microbial suspension, and again 500 nL of BGE in the 10-µL syringe. For CIEF separations, 4×10^{-2} mol L^{-1} sodium hydroxide and 0.1 mol L^{-1} orthophosphoric acid, both with addition of 3 % (v/v) EtOH and 0.5 % (w/v) Brij 35 or 0.5 % (w/v) PEG 10 000, were used as the catholyte and the anolyte solutions, respectively. Segmental injection into the capillary was employed in CIEF analysis [3]. The cell suspensions of *S. aureus* strains were adjusted to the concentrations from 5×10^{7} to 1×10^{8} cells mL^{-1}. Thiourea was used as a neutral marker of EOF at the measurements of a pH effect on the electroosmotic mobility, μ_{EOF}. The injection time ranged from 5 to 16 s.

3 Results and Discussion

In this contribution, we have demonstrated the utility of capillary zone electrophoresis and capillary isoelectric focusing as a fast and low-cost method to detect bloodstream infection with *Staphylococcus aureus*. Cells of *S. aureus* have a strong tendency to form agglomerates, a thorough sonication and vortexing of their samples were necessary before the separation.

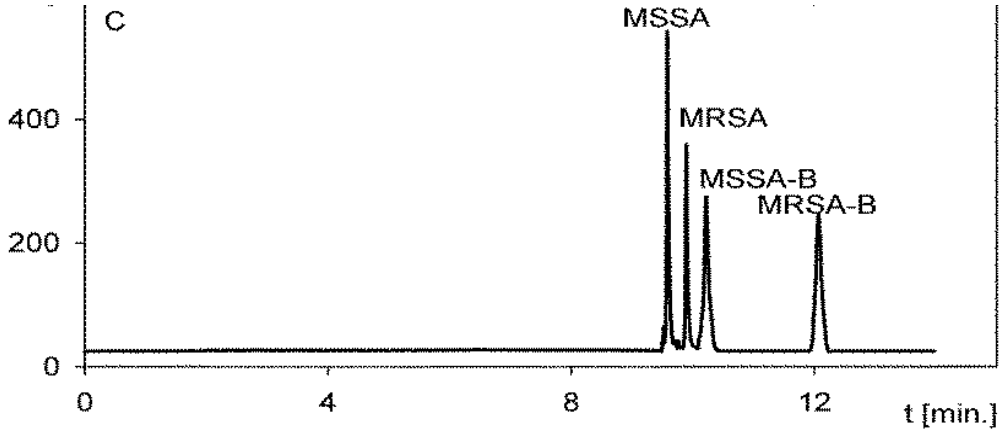

Fig. 1. CZE separation of *S. aureus* strains cultivated on MH agar (MSSA, MRSA) and *S. aureus* strains cultivated in blood (MSSA-B, MRSA-B).

4 Conclusions

Our results show a suitability of capillary electrophoresis to characterize and differentiate microorganisms due to their unique surface properties. Protocols for separation opportunistic pathogens bacteria methicillin-sensitive *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* were developed. We also verified the possibility to use these techniques for the quantification of the cell culture.

Acknowledgement

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P61 DIAGNOSTIC ACCURACY OF CYSTATIN C AND CREATININE BASED ESTIMATION OF GLOMERULAR FILTRATION USING VARIOUS EQUATIONS IN PEDIATRICS

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Introduction

The glomerular filtration rate (eGFR) is an important parameter for assessing renal function. The preferred methods for determining eGFR involve calculations using equations based on serum creatinine (Cr), cystatin C (CC) or a combination of these two biochemical markers without urine collection. In KDIGO 2012: Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease, the use of creatinine-based and cystatin C-based predictive equations is recommended in children [1]. Simultaneously, for eGFR in children, the creatinine-based prediction equations, e.g., Lund-Malmö [2], Counahan-Barratt [3], the cystatin C-based prediction equations, Hoek [4], Filler [5], Zapitelli [6] and the combined cystain C- and creatinine-based prediction equations, e.g. Zapittelli-CysCrEq [6] have been validated. The reason for using the combination of serum creatinine and cystatin C arose from the fact that the sources of error for either marker differ. Serum creatinine levels are distorted by muscle mass and variable tubular secretion, whereas serum cystatin C has a different volume of distribution and may vary with the volume status [7].

2 Objectives

The aim of the study was to compare different equations to estimate GFR in children and to select the most optimal equation.

3 Experimental

We examined a group of 133 children from the Nephrology Outpatients of the Department of Paediatrics of University Hospital Ostrava. Serum samples (gel tubes with clotting activator, Sarstedt, Nümbrecht, Germany) were used to analyse creatinine and cystatin C. Samples were centrifuged at 2050 g for 6 minutes, at 4°C and then analysed. Serum creatinine concentrations were obtained by means of the enzymatic method on the AU 5400 biochemical analyser (Creatinine Enzymatic, ref. OSR61204, Beckman Coulter, Inc., Brea, CA, USA) and serum cystatin C by

nephelometric assay on BN ProSpec (N Latex Cystatin C, ref. OQNM13, Siemens Healthcare Diagnostics Inc., Newark, DE, USA). Statistical evaluation of results was performed using QC Expert and R software.

Results and Discussion

In the study group of 133 children, 68 were boys (average age 11.44 years; SD 5.0) and 65 girls (average age 11.28 years; SD 5.56) from the Nephrology Outpatients of the Department of Paediatrics of University Hospital Ostrava. We examined six cystatin C-based, four creatinine-based and two creatinine-and cystatin C-based prediction equations. Table 1 shows very strong correlation between the concentration of cystatin C and the corresponding parameter-based eGFR equations.

Table 1. Results of correlations of cystatin C-based (CCEq), creatinine-based (CrEq) and cystatin C- and creatinine-based (CCCrEq) estimated GFR equation in the dependence on concentration of cystatin C (CC) and creatinine (Cr) using Pearson's (r_p) and Spearman's (r_s) rank correlation coefficient.

Parameter	**CC**		**Parameter**	**Cr**	
Correlation coefficient	**r_s**	**r_p**	**Correlation coefficient**	**r_s**	**r_p**
Hoek (CCEq)	-0.9997	-0.7938	**Lund-Malmö (CrEq)**	-0.4364	-0.5090
Levey (CCEq)	-0.9995	-0.7671	**Schwartz (CrEq)**	-0.8303	-0.4702
CKD-EPI (CCEq)	-0.9297	-0.8513	**Counahan-Barratt (CrEq)**	-0.8300	-0.4698
KDIGO (CCEq)	-0.9995	-0.8041	**CKD-EPI (CrEq)**	-0.9405	-0.7764
Filler (CCEq)	-0.9997	-0.7757	**CKD-EPI (CCCrEq)**	-0.7756	-0.7311
Zappitelli (CCEq)	-0.9996	-0.7693	**Zappitelli (CCCrEq)**	-0.7608	-0.6179

At the same time, all of the equations did not provide falsely high values of eGFR even at low values of cystatin C (Table 2, Figure 1). In the case of creatinine-based eGFR equations, the CKD-EPI equation correlated best with serum creatinine (r_s=-0.9405; r_p=-0.7764) and together with the Lund-Malmö equation did not provide falsely high values of eGFR (Table 3, Figure 1). The correlation coefficients of cystatin C- and creatinine-based equations were comparable and CKD-EPI gave better results than the Zappitelli equation when assessing the resulting values of eGFR (Table 3, Figure 1). The trueness of the equations for estimating eGFR values was assessed based on the median of all the equations. All medians of cystatin C-based equations for eGFR were not significantly different with the exception of the KDIGO equation, which gave up to 23% lower values of bias (Table 3). Of the creatinine-based equations, the CKD-EPI equation provided up to 30% higher values of bias (Table 3) despite the fact that its calculation is based only on the concentration of creatinine and patient age, while the Lund-Malmö equation, which also takes into account gender, provided much better results. At the same time, the Schwartz and Counahan-Barratt equations take into account the same parameters, creatinine

concentration and the patient's height and provide at low concentrations of creatinine comparable results with falsely positive values of eGFR (Table 3, Figure 1).

Table 2. Application of published on cystatin C-based prediction equations (CCEq) to testing data set of 133 children - basic statistical data.

			CCEq (ml/s per 1,73 m^2)					
	CC	**Cr**	**Hoek**	**Levey**	**CKD-EPI**	**KDIGO**	**Filler**	**Zappitelli**
Min.	0.446	8.3	0.36	0.27	0.29	0.35	0.36	0.28
Median	0.79	52	1.92	1.69	2.02	1.47	1.99	1.67
Mean	0.8385	52.76	1.926	1.708	1.956	1.47	2.004	1.683
Max.	3.65	309.2	3.45	3.34	2.74	2.5	3.78	3.26
Bias	-	-	0.52%	-11.52%	5.76%	-23.04%	4.19%	-12.57%

Table 3. Application of published on creatinine-based (CrEq) and cystatin C- and creatinine-based (CCCrEq) prediction equations to testing data set of 133 children - basic statistical data

	CrEq (ml/s per 1,73 m^2)				**CCCrEq (ml/s per 1,73 m^2)**	
	Lund-Malmö	**Schwartz**	**Counahan-Barratt**	**CKD-EPI**	**CKD-EPI**	**Zappitelli**
Min.	0.227	0.21	0.22	0.443	0.331	0.22
Median	1.602	1.73	1.82	2.466	2.179	1.78
Mean	1.617	1.889	1.981	2.549	2.233	1.872
Max.	3.084	10.92	11.45	5.469	3.541	5.19
Bias	-16.13%	-9.42%	-4.71%	**29.11%**	14.08%	-6.81%

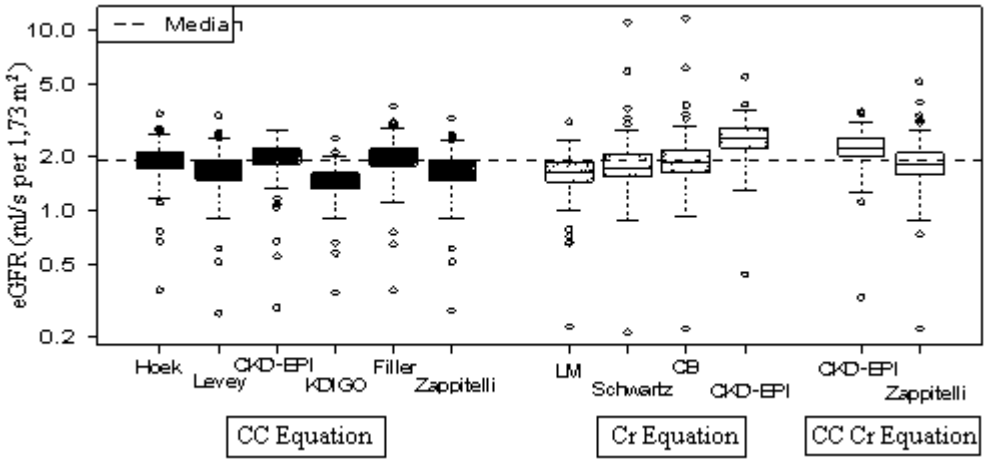

Fig. 1. Performance of six cystatin C-based (CC), four creatinine-based (Cr) and two creatinine-and cystatine C-based (CCCr) prediction equations for eGFR.

5 Conclusions

We conclude that the trueness of various cystatin C-based equations provides comparable GFR levels as do combined cystatin C- and creatinine-based equations.

Of the creatinine-based equations, the Lund- Malmö equation provides the best results. Still, when creatinine-based equations are applied for estimating GFR in individuals suffering from muscle-wasting caused by neuromuscular diseases, anorexia, liver cirrhosis, etc., the results must be interpreted with caution.

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P62 STUDY OF PROTEIN AND DRUG INTERACTIONS BY CAPILLARY ELETROFORESIS – MASS SPECTROMETRY

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Summary

Capillary electrophoresis hyphenated to mass spectrometry in frontal analysis mode (CE/FA-MS) was used to study the affinity between glimepiride and human serum albumin. Utilizing of both external and internal calibration methods for quantification of free drug concentration and subsequent determining of binding constants is presented in this contribution.

1 Introduction

Capillary electrophoresis hyphenated to mass spectrometry in CE/FA-MS mode presents an attractive technique for characterization of drug – protein interactions. The strength of interactions has a significant impact on both pharmacokinetics (i.e. absorption, distribution, metabolism and excretion) and pharmacodynamics (pharmacological effects) of corresponding drug. Among the advantages of the

CE/FA-MS method belong low demand on the sample amount, fast analyses and near-physiological conditions for studied interactions. MS detection offers enhanced sensitivity in comparison to common used UV detection. The increased sensitivity can be exploited for the determination affinity constants of poorly soluble compounds.

This study was focused on the study of interaction between anti-diabetic agent glimepiride (GLP) and human serum albumin (HSA). GLP is poorly soluble drug and belongs to third generation sulfonylureas used for treatment of diabetes mellitus type 2 because it acts as an insulin secretagogue.

2 Experimental

CE experiments were performed with the uncoated fused silica 50 cm long capillary with I.D. 75 μm. Operational voltage of 5 kV was applied in normal polarity and the temperature of capillary was maintained at 25 °C. 100 mM ammonium acetate solution, pH 7.4 was used as background electrolyte.

MS detection was performed with quadrupole – time of flight (Q-TOF) instrument. The electrospray MS measurements were conducted in the positive ionization mode. The sheath liquid consisted of MeOH-water and was delivered at a flow rate of 4 $\mu L \cdot min^{-1}$. The nebulizing gas pressure was set at 0.6 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.

Drug standard was dissolved in MeOH at concentration 500 μM. Samples of free drug and drug-protein mixture were prepared in the background electrolyte (BGE) solution, the organic solvent content in measured samples was below 2 %.

First CE/FA approach was based on the external calibration. It require off-line calibration curve – standards of drug without protein injected into the capillary and analysed. Than the drug-protein mixtures are analysed separately. Second approach used internal calibration. This approach utilizes single run measurements. Standard of the free drug is injected at first and followed by injection of drug-protein mixture and the analysis is performed, therefore off-line calibration curve is omitted [1]. Since analyses were done in a single run and measurement of calibration standard and affinity interaction was acquired under the same conditions, this approach should be more robust.

3 Results and Discussion

Analyses were performed using ammonium acetate BGE since it is both MS-friendly and close to physiological condition. Vuignier *et al.* found that HSA in phosphate and in ammonium acetate BGE showed the same binding percentages of drugs studied [2].

At first sample injection procedure was optimized. The injection time was evaluated in range 5 – 75 s with pressure 50 mbar and 50 s was found to be sufficient to obtain desired plateau of drug. In the case of analysis with the internal calibration injections of free drug and drug-protein mixture were done by pressure 50 mbar for 50 s and with BGE plug was injected between them. The BGE plug created by pressure 50 mbar at 60 s was found to be sufficiently long to separate them. Effect of injection volumes on plateau shapes was also considered.

Concentration of HSA was than optimized (20 – 0.1 μM) to obtain binding curves.

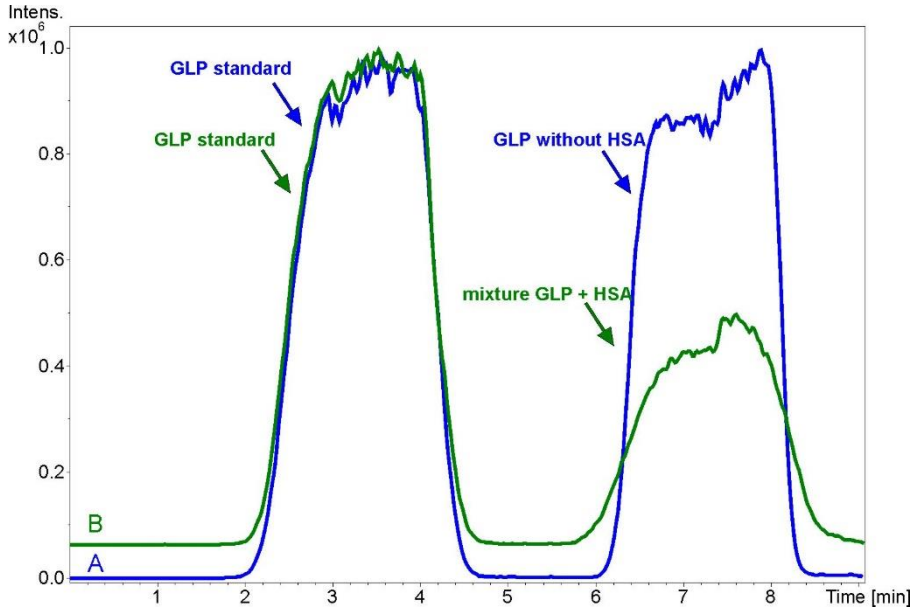

Fig. 1. Typical extracted ion electropherogram of CE/FA with internal calibration. A) Analysis of GLP standard followed by GLP standard. B) Analysis of GLP standard followed by GLP standard mixed with HSA.

4 Conclusions

CE/FA-MS provides analyses under near-physiological condition and it is well suited for measurement of binding constant. The main objectives of this study were to determine the binding constants for GLP and HSA using this CE based approach.

Acknowledgement

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P63 KINETIC INVESTIGATION OF HUMAN SALIVARY AMYLASE USING MICROCALORIMETRY

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1 Introduction

The environmental or genetic factors caused metabolic diseases become more common, but among them the incidence of diabetes is very significant. The better understanding of the functioning of carbohydrate modifying enzymes could reveal new targets, which contribute to the development of new effective therapies.

Isothermal titration calorimetry (ITC) is an increasingly used technique to investigate the molecular interactions but it is also appropriate for enzyme kinetic measurements, as well. In this study the ITC was used to determine the reaction rate of an enzyme catalysed hydrolysis reaction.

2 Experimental

Our study focused on human salivary amylase (HSA) and its inhibitors. The examination was carried out with ITC_{200} microcalorimeter. In the course of the measurement the heat change was followed as a function of reaction time. The differences between the baseline and the minimum point of the curve were considered as the reaction rate [1]. Plotting these rate values as a function of substrate concentration a saturation curve was obtained, which can be used to the determination of the enzyme kinetic parameters (K_M, V_{max}).

ITC was also used to characterize some known and new inhibitors. According to the rate values the kinetic constants (IC_{50}, K_i) and inhibition types was determined on the classical and a recent published way [2]. The examined inhibitors were acarbose, *Stevia rebaudiana* extract and glucopyranosylidene-spiro-thiohydantoin [3]. All the curve fittings were performed by Grafit program.

3 Results and Discussion

A set of method was established on the common, well-known HSA enzyme. According to the kinetic data, no significant differences were detected in the behavior of the free and the 4-nitrophenol chromophore containing maltoheptaose, in spite of the high number of the aromatic residues near the active site. The results of kinetic measurements with and without inhibitors are correlated well with previously published data.

4 Conclusions

This work is a preliminary study which forms the part of more enzyme including investigations to determine the main kinetic parameters and characterize the potential

inhibitors. The optimized methods are well applicable, thus it can serve as a good basis to our further examinations.

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P64 THE BIOANALYTICAL METHODS TO CHARACTERIZE ANTIFUNGAL AGENTS PRODUCED BY LACTIC ACID BACTERIA

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1 Introduction

The bioanalytical methods are commonly used for analysis of metabolites produces by microorganisms. The main interests of researchers are biologically active products, for example primary and secondary metabolites produced by lactic acid bacteria of the genus *Lactobacillus*. Antagonistic activity of *Lactobacillus* sp. resulting from the presence of these metabolites is directed mainly against the same type of bacteria but there is also activity against other bacteria [1, 2]. Furthermore, their antimicrobial properties are not associated with all species of bacteria of the genus *Lactobacillus* but only with theirs' particular cultures [3].

Studies show that antifungal activity of lactic acid bacteria is limited [4]. The major antifungal compounds (Fig. 1) produced by these bacteria are: the protein compounds with low molecular weight (less than 1000 Da), like reuterin (MW 148 Da; *Lactobacillus reuteri*) [5], 3-hydroxy fatty acids (MW 188 -244 Da; *Lactobacillus plantarum* MiLab) [5], phenyllactic acid (MW 166 Da, *Lactobacillus plantarum* 21B) [6], and cyclic dipeptides (MW 244-262 Da, *Lactobacillus plantarum, Lactobacillus coryniformis*) [7].

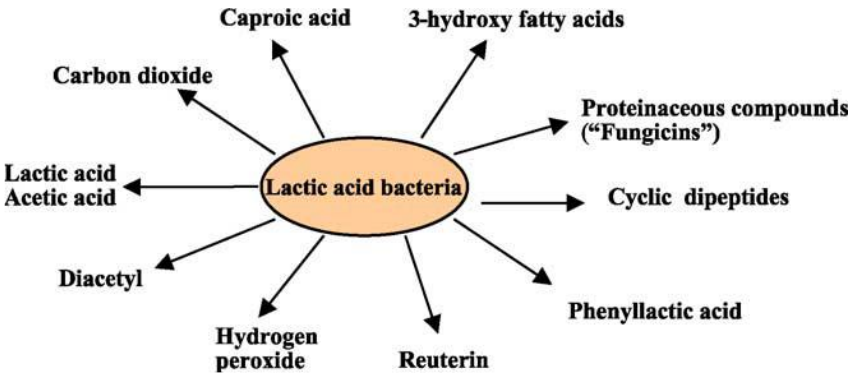

Fig. 1. Major antifungal compounds produced by lactic acid bacteria [8].

Knowledge about mechanism of anti-mold and anti-yeast activity of bacteria of the genus *Lactobacillus* can be useful to protect fermented food against undesirable microflora, especially pathogenic, toxin-creative and spoilage. Therefore, isolation and characterization of antifungal metabolites is important to find a way of preventing fungal contamination of food.

The chromatographic and spectroscopic methods give the opportunity to separate components of mixtures and to characterize them. In addition, enzymatic methods can be used to identification enantiomers of such chiral constituent as lactic acid which is a major product of lactic acid fermentation.

2 Experimental

Sixty strains of lactic acid bacteria of the genus *Lactobacillus* were screened for antifungal activity against eight strains of food-contaminating fungi (*Mucor hiemalis, Geotrichum candidum, Alternaria alternata, Alternaria brassicocola, Aspergillus niger, Fusarium latenicum* and two yeast strains of *Candida vini*). Antifungal activity of lactic acid bacteria was tested by a double layer method. Tested strains of bacteria were cultivated on the MRS agar (Merck) with different carbon sources (glucose, polyols, galactose) and tested fungi on the Sabouraud 4%-Dextrose agar (Merck). We selected a group of bacteria strains characterized by a broad-spectrum antifungal activity.

Next stage of experiment will be isolation and identification the metabolites of bacteria of the genus *Lactobacillus* after lactic acid fermentation using the methods: preparative chromatography, HPLS, HPLC-MS. Enzymatic methods will be use to distinguish between the enantiomers of chiral compounds.

3 Preliminary results and discussion

In the preliminary studies we have found that it is possible to use the bioanalytical techniques to isolate and characterize primary and secondary antifungal metabolites produced by lactic acid bacteria of the genus *Lactobacillus.* In addition to the high-performance liquid chromatography and mass spectrometry techniques, some researchers use also normal phase liquid chromatography combined with atmospheric pressure photoionization−tandem mass spectrometry (LC/APPI-MS/MS) [9], solid phase extraction (SPE) to separate one compound from others in the mixture and ion

chromatography (IC) to analyse for example organic acids and their antifungal properties [10].

4 Conclusions

The bioanalytical methods, especially chromatographic and spectroscopic, are useful do isolate and characterize antifungal agents produced by lactic acid bacteria. They provide support for more traditional methods used by microbiologists for increasing knowledge about mechanism of antifungal activity of lactic acid bacteria.

Acknowledgement

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P65 CITP ANALYSIS OF AMINOPOLYCARBOXYLIC ACIDS

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Summary
The new analytical method for the determination of content of aminopolycarboxylates by capillary isotachophoresis (CITP) with conductometric detection was developed. The qualitative and quantitative analysis of aminopolycarboxylates (*e.g.* NTA, EDTA) commonly present in mixture can be carried out with limits of detection about 10 μM and RSD about 5%. The developed method was validated by the analysis of industrial samples.

1 Introduction

Complexones are aminopolycarboxylic compounds capable of formation of stable complexes with majority of metal ions. EDTA, NTA and sodium salts are used in industry for their special properties. They are analyzed mostly by chromatographic methods [1]. CITP is an analytical electromigration technique which enables ion's separation based on their different mobilities and it can be used for determination of ionic analytes. The goal of this work was to verify the possible application of this technique for the migration study of zwitterionic compounds and their determination in mixture and in industrial samples.

2 Experimental

Method optimization for separation and determination of analytes was carried out on electrophoretic equipment EA 102 (Villa Labeco, Spišská Nová Ves, Slovakia) on PTFE capillary (diameter 0.3 mm, length 90 mm) joined with conductivity detector under laboratory temperature. Compounds (injection 30 μl) were analyzed in the following system: 20 mM HCl + glycylglycine (leading electrolyte, pH = 3.1), 20 mM acetic acid + NH_3 (terminating electrolyte, pH = 4.80). 0.5% hydroxyethylcellulose solution was added in order to eliminate electroosmotic flow.

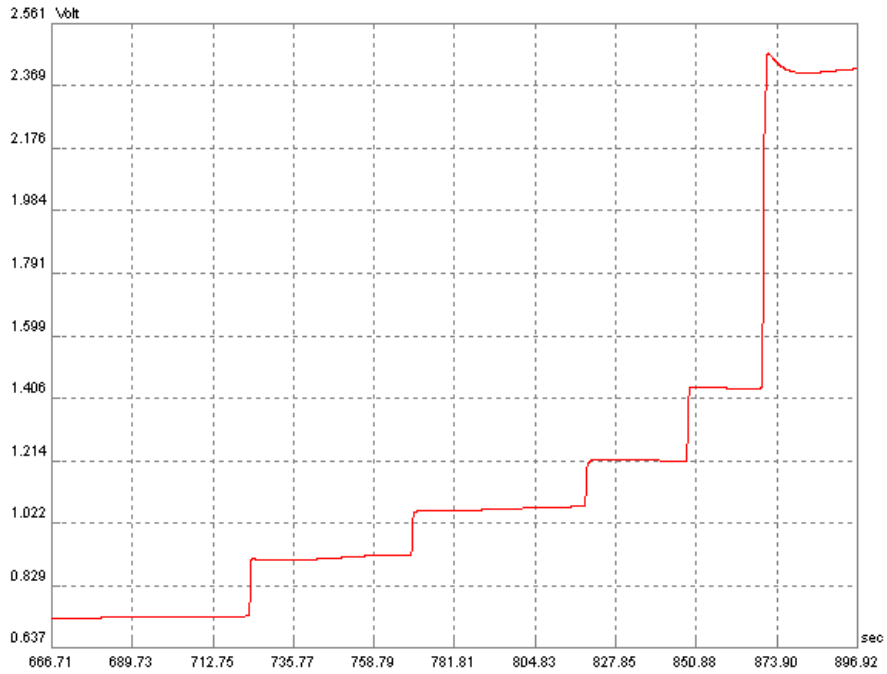

Fig. 1. The ITP record of analysis of aminopolycarboxylates (c = 0.5 mM, pH = 3.1), NTA, EDTA, IDA, GlyA – the order of analytes in ITP record).

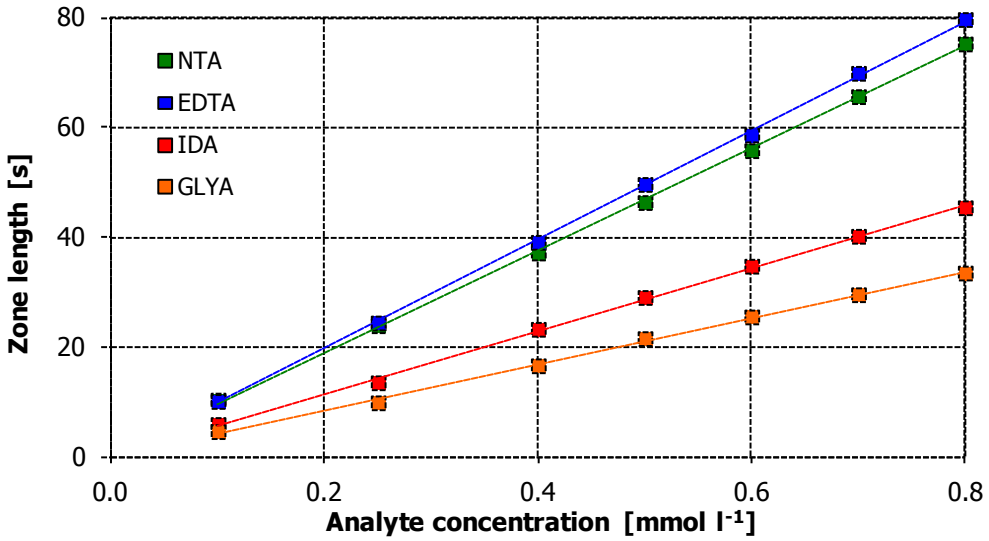

Fig. 2. Calibration plots for analyzed samples (see Fig. 2).

3 Results and discussion

The procedure was optimized by analysis of aminopolycarboxylates of known concentration in the mixture (see Fig. 1). The optimal pH = 3.1 was determined by mobility measurement in region pH = 2.5-4.5 when some mixed zones were observed. Detection limits of chosen analytes were determined from the calibration curves in concentration region 0.1-0.8 mM: NTA (0.06 mM), EDTA (0.05 mM), IDA (0.08 mM), glycolic acid GlyA (0.1 mM). This new analytical procedure was employed in the analysis of real industrial samples.

The mobilities of species of studied analytes were calculated from pH dependences and their estimates were used for simulation of ITP record (see Fig. 3). Comparing with experimental record (see Fig. 1), the agreement is evident.

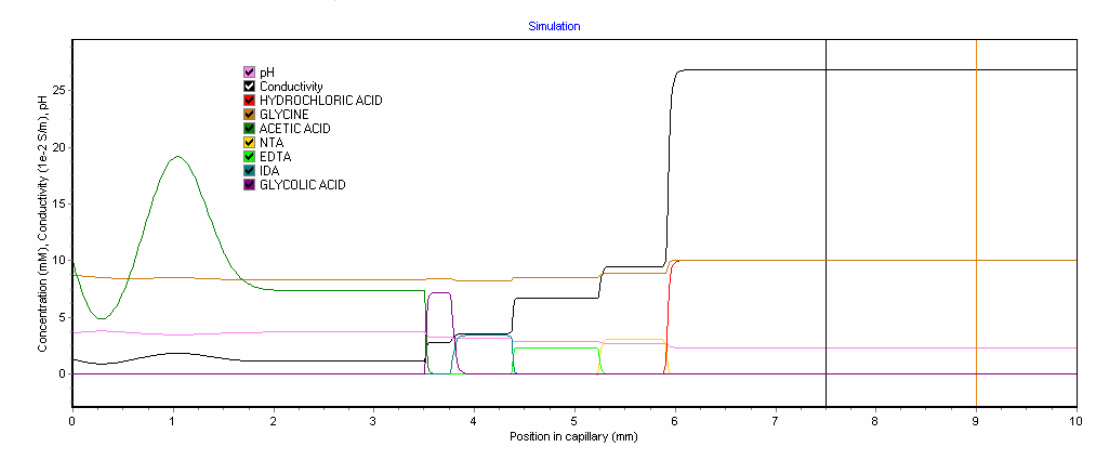

Fig. 3. The example of ITP record of mixture of analytes (c = 4 mM) simulated by means of SIMUL v 5.0 software (see ref. [2, 3]).

4 Conclusion

This contribution is focused on optimization of experimental conditions for determination of aminopolycarboxylic acids in industrial samples by means of CITP which is suitable alternative to other analytical methods.

Acknowledgement

The research was supported by the Ministry of Education of Czech Republic (grant **MUNI/A/0972/2013**), Grant Agency of Czech Republic (GA13-08336S) and EU programs (CEITEC CZ.1.05/1.1.0/02.0068).

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P66 CAPILLARY ELECTROPHORESIS WITH CONTACTLESS CONDUCTIVITY DETECTION FOR AMINO ACID PROFILING OF HUMAN PLASMA SAMPLES

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Summary
Amino acid (AA) profiling of plasma samples by means of capillary electrophoresis (CE) with contactless conductivity detection (C^4D) was performed. The suitability of CE-C^4D for the analysis of plasma samples was demonstrated by the analysis of plasma samples from patients diagnosed with cancer, coagulation defects, cystic fibrosis, and phlebitis. Statistical evaluation of data using ANOVA and PCA shown partial grouping of the samples according to the diagnosis.

1 Introduction

AA are crucial compounds to living organism. An altered metabolism of AA is distinctive for some inborn errors of metabolism [1]. AA profiles in plasma and urine differs in various types of cancer [2–5], chronic diseases [6,7], and they could contribute to the variation in response to drugs [8]. Analysis of AA in biological samples thus could give useful information about health-state of an organism to support the diagnosis and to understand the processes. CE is powerful analytical technique for the analysis of biological samples without laborious sample treatment and C^4D offers relatively sensitive detection of native AA. Application of CE-C^4D for AA profiling of plasma samples from patient of different diagnosis is presented.

2 Experimental

2.1 Chemicals and reagents

All chemicals were of the best purity available. Acetic acid (HAc), standards of AA, guanidineacetic acid (GAc) and hydroxyethyl-cellulose (HEC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN) was purchased from Burdick & Jackson (Honeywell International, Muskegon, MI, USA). Stock solution of 1 % (w/w) HEC was prepared at least a day prior use to assure proper hydration, and was intensively mixed using a magnetic stirrer.

2.2 CE-C^4D method

Agilent G7100 CE System (Agilent Technologies, Santa Clara, CA, USA) with integrated A/D converter was used in all experiments. C^4D was in-house made and inspired by *conductivity cell 2* introduced by Gaš *et al.* [9]. The working frequency of

the employed C^4D was 2.46 MHz. Background electrolyte (BGE) was comprised of 8 % (v/v) HAc and 0.1 % (w/w) HEC. BGE was filtered through nylon membrane filter with 0.45 μm porosity and degassed in an ultrasonic bath for 10 min. A bare fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 50/375 μm of inner/outer diameter and 80.0/65.6 cm of total/effective length was used. The capillary was rinsed before analysis by 1 M NaOH, 0.1 M NaOH, water and BGE for 3 min, 2 min, 3 min and 4 min, respectively. After analysis the capillary was rinsed by water for 3 min. Sample was introduced into the capillary by pressure 50 mbar for 30 s. The separation voltage and temperature were 30 kV (14 μA, 0.4 W) and 30 °C, respectively. Data acquisition and peak integration were accomplished by Agilent ChemStation software.

2.3 Plasma samples and sample treatment

Plasma samples were collected from patients suffering from i) malignant neoplasms of different topography (cancer); ii) coagulation defects, unspecified; iii) cystic fibrosis with pulmonary manifestation; iv) phlebitis and thrombophlebitis of unspecified deep vessels of lower extremities. The samples were collected from both sex of all ages. The plasma samples were stored frozen in a refrigerator at -20 °C. At the day of analysis, the plasma samples were thawed, mixed with ACN in volume ratio 1:2, thoroughly stirred and centrifuged at 14,000×g for 5 min. The supernatant was enriched by GAc giving final concentration 50 μM GAc and 3.1× diluted plasma sample.

3 Results and Discussion

51 plasma samples were analyzed and 18 AA were quantified in every sample. Results were grouped into 4 groups according to the diagnosis. One-way ANOVA was applied to analyze statistical difference among the groups for every AA. Seven AA were statistically different at the 0.01 level of significance (Arg, creatine, Glu, Gly, His, ornithine, Ser), 4 AA were statistically different at the 0.05 level of significance (creatinine, Glu, Lys, Phe) and 7 AA were not statistically different at the 0.05 level of significance (Ala, Ile, Leu, Pro, Thr, Tyr, Val) in respect of the groups. The levels of the AA, which were statistically different at the 0.01 level of significance, were normalized. The normalized levels of AA were submitted to Principal Component Analysis (PCA) using OriginPro 9 (OriginLab, Northampton, MA, USA) in respect of the diagnosis. The graph showing principal component scores of the PCA is depicted on Fig. 1.

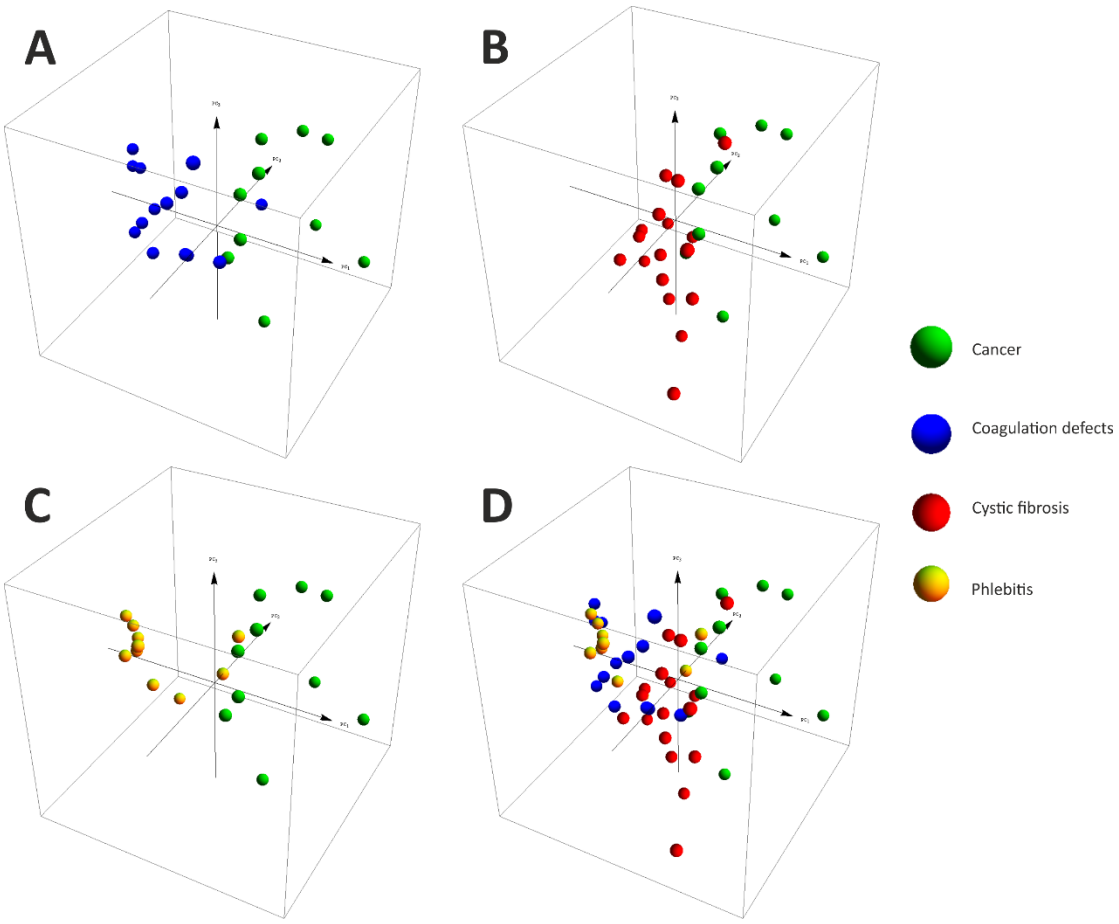

Fig. 1. The graph of the principal component scores of 51 plasma samples based on the levels 7 AA in the each sample (D). The graphs (A), (B), (C) showing principal component scores limited to only two groups of the diagnosis to facilitate the reader orientation in space.

4 Conclusions

The analysis of 51 plasma samples by CE-C^4D resulted in quantification of 18 AA in the all samples. The measured levels of AA were submitted to statistical analysis using one-way ANOVA and PCA. There was found that levels of 11 AA are statistically different at the 0.05 level of significance. PCA resulted in partial grouping of principal component scores in respect of the diagnosis. Presented CE-C^4D method could be considered useful for AA profiling of plasma samples in order to get supporting information about health-state.

Acknowledgement

Financial support granted by the Czech Science Foundation (Projects No. P206/11/0009 and P206/12/G014) is highly acknowledged.

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P67 ASSESSMENT OF AROMA COMPOUNDS IN APPLES USING SPME-GC-FID METHOD

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Summary
Twenty-five apple varieties, especially breed against apple scab (*Venturia inaequalis*) were analyzed for volatile aroma compounds content using SPME-GC-FID method. In total 60 various compounds were identified: 22 alcohols, 12 aldehydes, 11 ketones, 10 esters and 5 organic acids. Alcohols and aldehydes were the most abundant compounds in most varieties. The total content and single chemical groups of compounds were then compared with standard variety (Golden Delicious). Significant differences ($p < 0.05$) were found among samples, depending on species, cultivation and breeding.

1 Introduction

Apple trees (*Malus domestica*) belong to the most widely spread, important and valuable fruit [1]. The composition of apples is very variable, depending on many known varieties; saccharides (glucose and fructose) are the main components, greatly influencing overall sensory quality of fruit. The fruit is rich in vitamins, especially vitamin C and also trace elements including Co, Cu, Mg, Zn and Mo [2]. Apples are universally used, such creating a significant proportion of harmonious human nutrition. Its therapeutic value is well known for different illnesses (e.g. cancer,

atherosclerosis, diarrhea, bowel inflammation). Much of the crop is processed into canned fruit, syrups, ciders, marmalades, wines and liquors [3]. The aroma profile of apples has been characterized by a few authors before [4 – 6]. It contains more than 300 different compounds, most of them are C5 acids, alcohols and esters [7].

The aim of this study was to identify, quantify and compare the volatile aroma compounds of selected species of apples. The volatiles were extracted by solid phase microextraction (SPME) and assessed by gas chromatography with flame ionization detection (GC-FID).

2 Experimental

2.1 Samples

Twenty-five species (genotypes) of apples, especially breed against apple scab (*Venturia inaequalis*) were analyzed (sample labeling see Fig. 3.1), including Golden Delicious (GD) (Research and Breeding Institute of Pomology Holovousy Ltd.), as standard. The varieties were harvested in September 2012 and stored in a freezer until analysis.

2.2 SPME-GC-FID conditions

Volatile compounds were extracted by SPME, identified and quantified using standards by GC-FID. The SPME conditions were: SPME fiber CAR^{TM}/PDMS 85 μm (Supelco). Sample volume 1 g, extraction temperature 35 °C, equilibrium time 30 min., extraction time 20 min., desorption temperature 250 °C, desorption time 20 min. Gas chromatograph used was TRACE^{TM} GC (ThermoQuest, I), capillary column DB-WAX (30 m × 0,32 mm × 0,5 μm). GC conditions: injector 250 °C, splitless desorption 5 min, carrier gas N_2 0,9 ml·min^{-1}, FID at 220 °C, H_2 35 ml·min^{-1}, air 350 ml·min^{-1}, make-up N_2 30 ml·min^{-1}. The oven temperature was 40 °C for 1 min., 40 – 200 °C at 5 °C·min^{-1}, 200 °C for 7 min. Total analysis time was 42 min. The results were statistically treated using one way Wilcoxon test at $p < 0.05$ using Unistat version 5.5.

3 Results and Discussion

In total 60 various aroma compounds were identified in the samples: 22 alcohols, 12 aldehydes, 11 ketones, 10 esters and 5 organic acids. The comparison of the total content of compounds identified in single varieties is expressed in Fig. 1, the comparison of group of compounds in Fig. 2. As can be seen in both Figures, there are significant differences ($p < 0.05$) among varieties. Fig. 1 shows that variety HL 898 greatly exceeds the others with total concentration of aroma compounds 394.77 ± 2.38 μg·g^{-1}. The second highest amount was measured in HL 345 (297.67 ± 1.71 μg·g^{-1}), followed by HL 322 (253.61 ± 2.95 μg·g^{-1}). The lowest total concentration was found in HL 164 (38.66 ± 0.43 μg·g^{-1}). Golden Delicious belongs to the varieties with mild aroma (confirmed by sensory evaluation), that's why the content of aroma compounds was quite low (86.90 ± 0.82 μg·g^{-1}). Nine evaluated apple varieties contained even higher ($p < 0.05$) amount than standard.

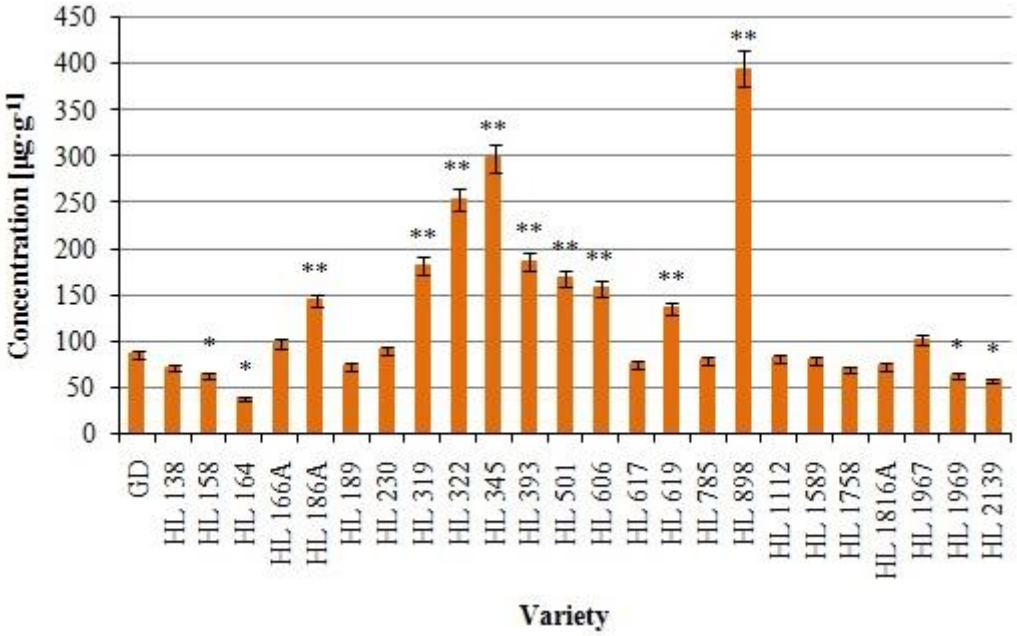

Fig. 1. Total concentration of aroma compounds identified in apple varieties (µg·g^{-1} per sample).

* significantly ($p < 0,05$) lower concentration than standard

** significantly ($p < 0,05$) higher concentration than standard

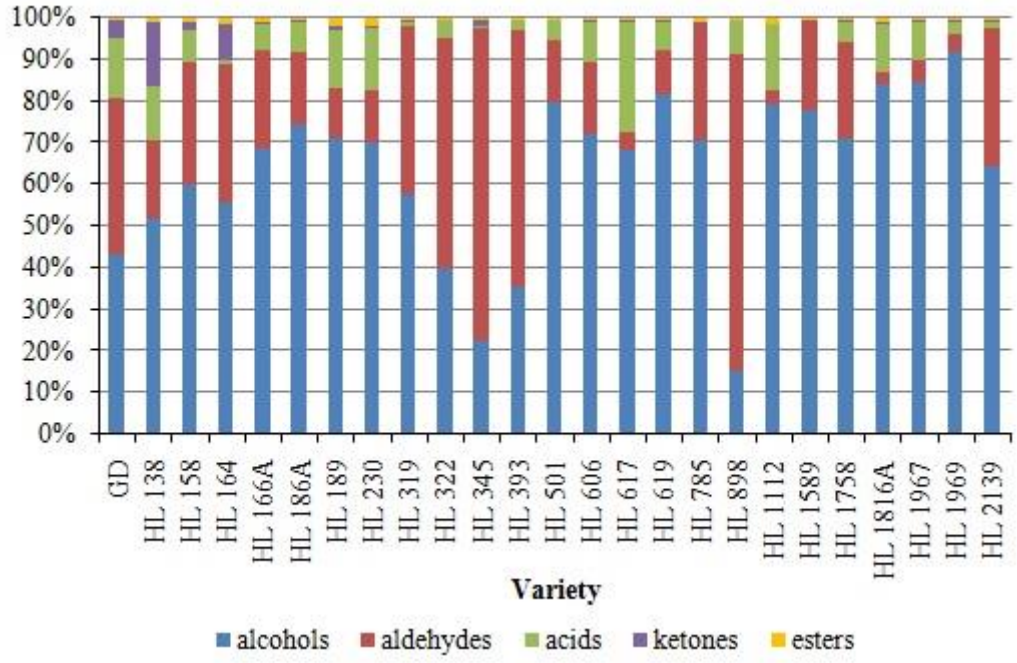

Fig. 2. Percentage of the chemical groups identified in apple varieties.

Fig. 2 indicates that alcohols create the highest percentage of the chemical groups in almost all varieties. Aldehydes are the second most important group, which dominate in four varieties. Ketones and esters are represented minimally.

4 Conclusions

SPME-GC-FID method was applied on the selected cultivars of apples resistant to apple scab. Great differences were found among samples, with the specific individual composition and content of aroma compounds in single varieties, depending on species and their cultivation and breeding.

Acknowledgement

We are grateful to prof. Řezníček from Mendel University in Brno and Research and Breeding Institute of Pomology Holovousy Ltd. for providing samples for the study.

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P68 DETERMINATION OF BINDING CONSTANT OF 1ST GENERATION ANTIDIABETIC DRUG WITH HUMAN SERUM ALBUMIN

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Summary

The capillary electrophoresis fontal analysis (CE-FA) was used to study the affinities between selected 1st generation antidiabetic drugs (tolbutamide, chlorpropamide) and human serum albumin (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 (RSD < 3%).

1 Introduction

The binding constant (K_b) is commonly used to describe the strength of binding between ligand such as drug and protein. The strength of the interaction has a

significant effect on biological activity of the drug [1]. Various sulfonylureas (antidiabetic drugs) may induce hypoglycemia as a result of excesses in insulin production and release [2]. Therefore, it is important to understand in detail the pharmacokinetics and pharmacodynamics of these drugs. 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 constants of the selected drugs – tolbutamide, chlorpropamide with HSA using CE-FA under physiological conditions.

2 Experimental

2.1 Chemicals

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

The BGE in all experiments was the phosphate buffer, pH 7.4 (I = 0.002) [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) or 200 nm (chlorpropamide).

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 both drugs.

3.2 Determination of binding constants

Optimized method was used to determination of binding constants of two model systems tolbutamide-HSA and chlorpropamide-HSA. The set of representative binding curves obtained for tolbutamide – HSA with CE-FA method is shown in Fig. 1. CE-FA provides good repeatability, RSD of log K_b value is lower than 3 %.

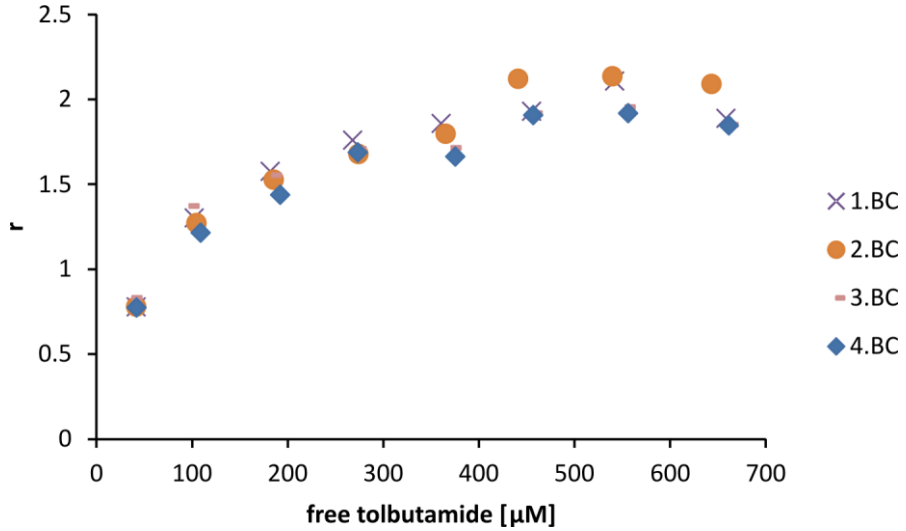

Fig. 1. Repeatability of binding curves (BC) obtained for tolbutamide – HSA system with the CE-FA method. Experimental conditions: BGE - phosphate buffer, pH 7.4, hydrodynamic injection (5 s, 35 mbar), separation voltage – 10 kV, λ = 214 nm, samples - mixture of 75 µM HSA with 100-800 µM tolbutamide.

4 Conclusions

The main aim of this work was to develop a method for K_b determination of tolbutamide/chlorpropamide and HSA. Apparent K_b values obtained using nonlinear regression were $1.34 \pm 0.36 \cdot 10^4$ L/mol for tolbutamide and $9.71 \pm 2.42) \cdot 10^3$ L/mol for chlorpropamide.

Acknowledgement

This work was supported by grant No. P206/12/G014 from the Czech Science Foundation.

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P69 DETAILED STUDY OF IMATINIB METABOLISM USING HIGH-RESOLUTION MASS SPECTROMETER ORBITRAP ELITE

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1 Introduction: Therapeutic drug monitoring is widely applied useful tool for treatment individualization in order to achieve optimal clinical response and avoid toxicity. Due to drug metabolizing enzymes several bioactive or toxic metabolites are produced. Therefore determination of parent drug and also of metabolites may have clinical relevance and could be helpful for treatment adjustment. The aim of this study was to assess a detailed profile of imatinib (IM) metabolites in plasma of patients with chronic myeloid leukemia (CML).

2 Experimental

Plasma proteins were precipitated by methanol and supernatant was used for analysis. Separation proceeded on Phenomenex Kinetex C18 column (100 x 2.1 mm; 1.7 µm) using UltiMate 3000 RS (Thermo Scientific) liquid chromatography with mass spectrometry detection using Orbitrap Elite instrument (Thermo Scientific) based on exact mass measurement. Scan range of m/z 350 – 1200 was chosen and the resolution was set at 60,000 FWHM. Fragmentation spectra were collected within m/z 135 – 800 at resolution 30,000 FWHM. All measurements were performed with mass accuracy < 5 ppm. Data were evaluated using Excalibur 2.2 SP1, MetWorks 1.3 SP3 and Mass Frontier 7.0 software.

3 Results and Discussion

In eight plasma samples of CML patients with standard IM dose 400 mg/day and sampling time of 24±4 hours after last dose (steady state) 90 metabolites in concentration range of 0.1 nmol/L - 1 µmol/L were found. For confirmation of metabolite identities characterized by *m/z* values, MS^2 exact mass fragmentation of phase I metabolites and MS^3 fragmentation of phase II metabolites (conjugates) in orbitrap mass analyser were performed. Fragments from MS^2 and MS^3 experiments were compared with theoretical predicted fragments generated by Mass Frontier 7.0

software. In our experiment three main fragments of IM with abundance higher than 1% were observed: m/z 394.1662, 217.1335 and 476.2557. Other three low abundant fragments are present in spectra: m/z 189.1386; 396.1819; 174.0913. Differences in metabolic profiles between two plasma samples of selected patients were observed. Compared to IM there are differences in relative intensities of demethylation, oxidation, demethylation+oxidation and dioxidation. Further, samples are different in overall intensities of all metabolites of selected metabolisations.

4 Conclusions

New generation of high resolution mass spectrometry offers sensitive tool for detail study of drug metabolism in biofluids. IM is transformed to several metabolites that could serve as useful diagnostic tool. Further studies should be focused on elucidation of clinical significance of newly discovered metabolites.

Acknowledgement

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P70 LIQUID CHROMATOGRAPHIC MASS SPECTROMETRIC DETECTION OF METABOLITES OF MINOR BENZO[*C*]PHENANTHRIDINE ALKALOIDS

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1 Introduction

Quaternary benzo[*c*]phenanthridine alkaloids (QBAs) belong to a group of substances obtained from plants of families *Papaveraceae*, *Fumariaceae* and *Rutaceae*. While the major and commercially available sanguinarine (SA) and chelerythrine (CHE) have been involved in biological and pharmacological studies over recent years [1,2], metabolic data on minor QBAs including pentasubstituted sanguilutine (SL) and hexasubstituted macarpine (MA) (Figure 1) are limited, due to their slow accumulation in plant material as they represent the end products in their respective biosynthetic pathways. Biological activity includes pro-apoptotic, antitumor, antiinflammatory, antimicrobial, antifungal and antiparasitic effects. As potential

drugs, their toxicity should be determined prior any commercial application. One of the substantial parts of the pharmacokinetic and toxicity studies is determination of their metabolic fate. Enzymes of endoplasmatic reticulum, located on rat liver microsomes (RLMs), such as cytochrome P450, may serve as a simple *in vitro* model for metabolic studies. The metabolites can be analyzed by high performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (MS).

Despite the very similar structure of QBAs, metabolic pathways may differ considerably. Results published on metabolites of SA and CHE indicate demethylation, hydroxylation and ring-opening reactions.

Alkaloid	R_1	R_2	R_3	R_4	R_5	R_6
Sanguinarine (SA)	$-CH_2-$		$-CH_2-$		-H	-H
Chelerythrine (CHE)	$-CH_2-$		$-CH_3$	$-CH_3$	-H	-H
Sanguilutine (SL)	$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$	$-OCH_3$	-H
Chelilutine (CL)	$-CH_2-$		$-CH_3$	$-CH_3$	$-OCH_3$	-H
Sanguirubine (SR)	$-CH_3$	$-CH_3$	$-CH_2-$		$-OCH_3$	-H
Chelirubine (CR)	$-CH_2-$		$-CH_2-$		$-OCH_3$	-H
Macarpine (MA)	$-CH_2-$		$-CH_2-$		$-OCH_3$	$-OCH_3$

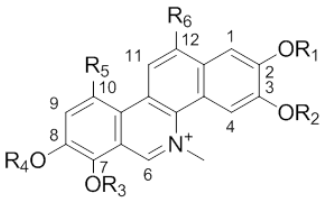

Fig. 1. Quaternary benzo[*c*]phenanthridine alkaloid structures.

2 Experimental

2.1 Extraction and isolation of QBAs

SL, and MA were isolated according to a method described by Slavik *et al.* [3] Isolation and purification step was performed by means of semi-preparative reversed-phase (RP) chromatography. The purity of alkaloids was > 90 %.

2.2 Isolation of RLMs

Male rats (*Wistar albino*) aged 8 weeks were sacrificed by decapitation and subjected to laparotomy. A liver sample (4 g) was homogenized and the microsomal fraction was prepared by differential centrifugation in a 20 mM Tris/KCl buffer (pH 7.4) and washed with 0.15 M KCl, according to conventional methods. The total protein was determined by the Lowry method using bovine serum albumin as standard. [4] Aliquots were stored at –80 °C until needed.

2.3 Incubation, extraction and analysis of samples

Each alkaloid (50 μL, 18 μM) was incubated at 37 °C with 50 μL of RLMs in a buffered (50 mM phosphate, pH = 7.4) NADPH generating system (glucose-6P dehydrogenase, glucose-6P) in total volume of 1 mL. The reaction was stopped by an addition of 100 μL of ice-cold methanol and transferring to an ice bath.

Metabolites were purified by adding 400 μL of MeOH:ACN:formic acid (50:50:0.02 *v/v/v*) into a 100 μL aliquot of the incubation mixture, followed by vortex and centrifugation. A portion (500 μL) of the supernatant was evaporated under a stream of N_2 at room temperature and the residue (~50 μL) was diluted with water (130 μL) and injected (20 μL) onto an LC column.

2.4 LC MS

LC MS method was established using a Dionex Ultimate 3000RS (Thermo Scientific, Sunnyvale, CA) module. Compound separation was achieved with a 3.0 × 150 mm, 4μm Synergi RP-Max C18 column at 23 °C and a flow rate of 0.5 mL/min. A binary mobile phase system consisted of 0.1% formic acid (A) and LC-MS grade ACN (B). Mobile phase B was increased from 20 % to 40 % for 10 min and then to 80 % for next 10 min. It was kept constant for 10 min followed by equilibration to the initial conditions for 3 min. A total HPLC run was 33 min. The HPLC system was connected to a MicrOTOF-QII (Bruker, Germany) mass spectrometer, operated in positive electrospray ionization mode. The ionization conditions were set by the software as the following: Capillary voltage 4500 kV, end plate offset –0.5 kV, source temperature 220 °C, desolvation gas (nitrogen) flow 8 L/min, nebulizer (nitrogen) pressure 3 bar, collision cell voltage 35 eV.

Table 1. Retention time, exact mass, elemental composition and type of transformation of SL and MA after 120 min incubation with RLMs.

Alkaloid	Detected compounds	t_r (min)	Ion	Observed m/z	Sum formula [M]	Mass shift (Da)	Reaction type	
SL		6.9	$[M]^+$	394.1654	$C_{23}H_{24}NO_5$	0		
	O-Demethyl-SL	5.6	$[M]^+$	380.1498	$C_{22}H_{22}NO_5$	-14	$-CH_2$	O-demethylation
	Hydroxy-SL	6.1	$[M]^+$	410.1604	$C_{23}H_{24}NO_6$	16	+O	monooxygenation
	Bis(O-demethyl) dihydro-SL (A)	6.7	$[M+H]^+$	368.1499	$C_{21}H_{21}NO_5$	-26	$-CH_2-CH_2+2H$	reduction, O-demethylation (2x)
	Bis(O-demethyl) dihydro-SL (B)	8.5	$[M+H]^+$	368.1487	$C_{21}H_{21}NO_5$	-26	$-CH_2-CH_2+2H$	reduction, O-demethylation (2x)
	O-Demethyl dihydro-SL (A)	12.5	$[M+H]^+$	382.1668	$C_{22}H_{23}NO_5$	-12	$-CH_2+2H$	reduction, O-demethylation
	O-Demethyl dihydro-SL (B)	13.1	$[M+H]^+$	382.1639	$C_{22}H_{23}NO_5$	-12	$-CH_2+2H$	reduction, O-demethylation
	O-Demethyl dihydro-SL (C)	14.1	$[M+H]^+$	382.1639	$C_{22}H_{23}NO_5$	-12	$-CH_2+2H$	reduction, O-demethylation
	Oxy-SL	16.5	$[M+H]^+$	410.1604	$C_{23}H_{23}NO_6$	16	+O	monooxygenation
	Hydroxy dihydro-SL	17.0	$[M+H]^+$	412.1759	$C_{23}H_{25}NO_6$	18	+O +2H	reduction, monooxygenation
	Dihydro-SL	18.5	$[M+H]^+$	396.1853	$C_{23}H_{25}NO_5$	2	+2H	reduction
	Nor-SL	19.2	$[M+H]^+$	380.1503	$C_{22}H_{21}NO_5$	-14	$-CH_2$	N-demethylation
MA		7.8	$[M]^+$	392.1169	$C_{22}H_{18}NO_6$	0		
	Demethylene-MA	5.1	$[M]^+$	380.1122	$C_{21}H_{18}NO_6$	-12	–C	demethylenation
	Demethylene dihydro-MA	11.2	$[M+H]^+$	382.1283	$C_{21}H_{19}NO_6$	-10	–C +2H	reduction, demethylenation
	Dihydro-MA	22.3	$[M+H]^+$	394.1319	$C_{22}H_{19}NO_6$	2	+2H	reduction
	Nor-MA	23.3	$[M+H]^+$	378.0989	$C_{21}H_{15}NO_6$	-14	$-CH_2$	N-demethylation
	Oxy-MA	18.4	$[M+H]^+$	408.1090	$C_{22}H_{17}NO_7$	16	+O	monooxygenation

3 Results and Discussion

The identified metabolites are summarized in Table 1. The elemental formula of the metabolites was obtained from the accurate *m*/*z* and their structure was deduced from MS/MS spectra and retention behavior. Observed metabolic reactions include reduction to dihydro-derivatives (+2 Da), O-demethylation (–14 Da), hydroxylation (+16 Da), demethylenation (–12 Da), N-demethylation (–14 Da), and/or a combination of these processes.

4 Conclusions

No demethylation of MA was observed in opposition to SL where it was a dominant type of transformation. This might be caused by a preferential loss of a methylene group from the MA structure or by an ability of MA acting as an inhibitor of one or more enzymes from the cytochrome P450 complex. This may be inspected by introducing a selective inhibitor into the reaction mixture prior to incubation.

Acknowledgement

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P71 OPTIMIZATION OF SARCOSINE OXIDASE BIOSENSOR FOR DETERMINATION CARBOXYLIC ACIDS IN REAL SAMPLES OF FRUIT WINE

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Summary

The flow-through one-channel amperometric biosensor for monitoring of relative inhibition of sarcosine oxidase (SOX) was presented. Biosensor was based on two sensing layers deposited on a screen-printed platinum electrode. The inner layer eliminated interferences by limiting diffusion of electrochemically active substances such as ascorbic acid and polyphenols. This layer was formed by electropolymerization using the equimolar mixture of *o*-phenylenediamine and resorcinol in 2 mM or 4 mM concentration. The outer layer was prepared by cross-linking the enzyme SOX and bovine serum albumin (BSA) using glutaraldehyde (GA). The formation of enzymatically produced hydrogen peroxide was monitored at 650 mV vs. an Ag/AgCl reference electrode. The addition of carboxylic acids caused competitive reversible inhibition of the immobilized enzyme and a decrease in signal. The assay was optimized for determination of carboxylic acids in fruit wine samples. The 10-fold dilution of real samples and a 5 mM concentration of sarcosine was chosen as optimal for competition. In case of real samples, the biosensor measured the sum of all carboxylic acids, which served as a parameter describing the quality of fruit wines. Two types of fruit wines including bilberry and chokeberries were used for biosensor optimization. To better understand the influence of phenolic compounds during sample measurement, the solid phase extraction (SPE) for elimination of phenolic compounds was used.

1 Introduction

The fruit wine represents quite complex fluid consisting of mixture acids, sugars, alcohols, polyphenols and other minor compounds. These components comprehensively affect taste, aroma and other characteristics of the prepared fruit wine. Carboxylic acids are main components in fruit wine and they are responsible for a large degree of acidity and tartness in wine. The composition of fruit wine is dependent on the species of the originally used fruit. The measurement of organic acids is important due to its high content in fruit wines. There are a lot of techniques for determination organic acids in wine [1]. Mainly, carboxylic acids cointained in wine are determined using standardized methods including HPLC [2,3] and capillary electrophoresis [4,5]. The enzymatic methods for the detection of carboxylic acids were also introduced [6].

Our effort was aimed to utilizing the developed biosensor for carboxylic acids determination. We focused on sample pretreatment where we monitored the behaviour of biosensor in the presence of interference compounds.

2 Experimental

2.1 Materials

Sarcosine oxidase (SOX, from *Bacillus* sp., 25–50 units·mg^{-1}), sarcosine, bovine serum albumin, o-phenylenediamine, resorcinol, glutaraldehyde (GA), were purchased from Sigma. Sodium hydrogenphosphate, sodium dihydrogenphosphate, potassium chloride, methanol, diethyl ether and hydrochloric acid and hydrogen peroxide were purchased from Penta (CZE). SPE column Oasis HLB 12cc (500mg) extraction cartridge was purchased from Waters Corporation (USA).

2.2 Pretreatment of fruit wine

The SPE column was activated with methanol and washed with distillated water at first, the treatment of the sample followed and the last step was regeneration of SPE column with methanol, gaseous N_2, diethyl ether and hydrochloric acid.

2.3 Polymerization proces

The working electrode was coated with a thin layer of the selective noncondutive copolymer (2mM / 4mM *o*-phenylenediamine and 2mM / 4mM resorcinol) which grows until its maximum thickness. As the surface becomes isolated, electric current decreases in relation with increasing polymer thickness.

2.4 Biosensors

Enzyme layer was prepared by cross-linking of SOX (5 U.cm^{-2}) with 2% GA in 50 mM phosphate (pH 7.4). The mixture was supplemented with 100 mg.ml^{-1} BSA in order to reach the density of 2 mg.cm^{-2}. The immobilization mixture was deposited on the copolymer-coated working electrode and stored at 4°C.

3 Results and Discussion

Despite the fact that the formed copolymer was able eliminate the nonspecific response of ascorbic acid, the real sample measurement showed the increase of nonspecific response. This increase corresponded to lowering of relative inhibition of biosensor. The adding the SPE pretreatment step to the measurement procedure should help to eliminate this negative behavior. In this case phenolic compounds were effectively removed using the SPE column. We monitored the biosensor responses with or without of this pretreatment step.

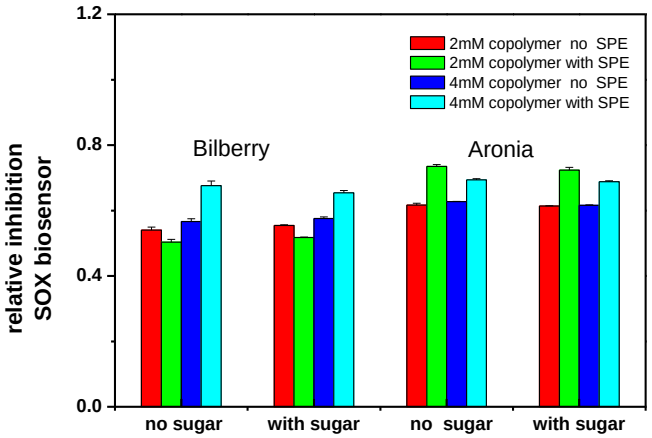

Fig. 1. Relative inhibition of SOX biosensor with different concentration of copolymer - the effect of SPE.

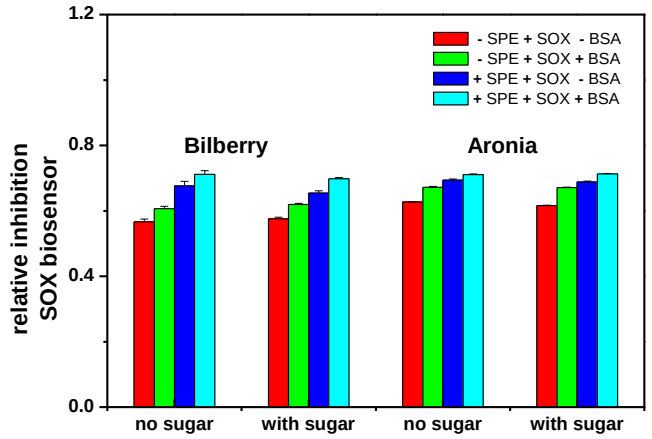

Fig. 2. Realitve inhibition of SOX biosensor with 4 mM copolymer layer - the effect of BSA in the biosensing layer.

The influence of the sugar added during fermentation of fruit wine on signal of the relative inhibition was not detected. Increased concentration of the polymer significantly affects response of signal - the relative inhibition in both fruit wines. The evident improvement of the relative inhibition using SPE column was observed.

To increase the relative inhibition of the SOX biosensor, there was a significant contribution to the use of SPE column and BSA modified biosensing layer. Very high signal, for the model SOX biosensor consisting of a polymer (4 mM) and BSA modified layer and pretreatment process with SPE column, both of fruit wine has been observed.

Based of these results, we evaluated which type of electrode is the best for measurement for all real samples of fruit wine, which electrode can provide a good diffusion processes (H_2O_2) through the membrane, and at the same time to be a good protector of the electrode from chemical intereferents and to show the highest relative inhibition value. With analysis of fruit wines we obtained that the SOX biosensors in

combination with copolymer (4mM) and SPE colon and biosensors of BSA shown very good results (more then 70%) in the relative inhibition of SOX biosensor.

4 Conclusions

The phenolic compounds significantly influenced the behaviour of biosensor. The formed copolymer layer insufficiently eliminated the electrochemical activity of phenolic compounds which corresponded to lower inhibiton of the biosensor. Using SPE for removing of phenolic compound together with diferential measurement using SOX and BSA modified biosensors improved the inhibition effect of biosensor about 17%. This efect was not observed in the case of grapes wine due to the lower total content of phenolic compounds in comparison with fruit wines.

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P72 DETERMINATION OF ALIPHATIC CARBOXYLIC ACIDS IN PARTIALLY FERMENTED GRAPE MUST

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1 Introduction

One of the major factors affecting the quality of wine is the content of organic (aliphatic and aromatic) acids. Aliphatic acids normally present in wine are tartaric acid, malic acid, oxalic acid, citric acid, lactic acid, acetic acid, and malonic acid. Winemaking is a lengthy process, which consist of several steps. It starts with the grape harvest, then production of must and finally the formation of wine [1]. Partially fermented grape must is one of the intermediates in the wine production process and that's why we chose it as our sample. Ion-exclusion chromatography (IEC) is the chromatographic technique, which has been used for the separation of relatively small acids, weak bases and hydrophilic molecular species [2]. The separation mechanism is based on the separation of partially dissociated analytes on a strong cation exchanger.

Completely dissociated strong acids are repelled by the positively charged cation-exchange functional groups that make up the so-called Donnan membrane. Undissociated or partially dissociated weak acid pass through the membrane and are thus retained on the column longer. The separation is determined by the IEC Donnan repulsion, as well as steric exclusion and sorption processes [3]. The aim of this work was to develop a simple method for the determination of aliphatic acids in partially fermented grape must by IEC with spectrophotometric detection.

2 Experimental

2.1 Instruments

Chromatograph Elite LaChrom (Merck - Hitachi, Darmstadt, Germany) with a diode array detector (L-2450) was used to separate a mixture of standard acids and to determine acids in a sample of the partially fermented grape must.

The separation was carried out on the analytical column Alltech Prevail^{TM} organic acid 5 μm (150 mm × 4.6 mm I.D) with a guard column Prevail 5 μm organic acid (7.5 × 4.6 mm I.D) (Grace - Deerfield, USA). Further for the preparation of solutions the AR 0640 analytical balance (Ohaus, USA), pH meter Agilent Technologies 3200P (Agilent Technologies - California, USA), ultrasonic bath UCM9 (Ecoson - Nové Mesto, Slovak Republic) were used.

2.2 Chemicals and standard solutions

All chemicals were of analytical reagent grade and were purchased from Merck (Darmstadt, Germany). Stock solutions of aliphatic acids had the following concentrations: succinic acid, malonic acid, malic acid, oxalic acid, tartaric acid, citric acid, acetic acid, propionic acid, lactic acid and itaconic acid 10 000 $mg \cdot L^{-1}$, maleic and fumaric acids 1000 $mg \cdot L^{-1}$. Calibration solutions were prepared by dilution of the stock solutions and the ranges are in Table 1. The mobile phase was prepared from a 25 $mmol \cdot L^{-1}$ solution of potassium dihydrogen phosphate, the pH was adjusted with concentrated phosphoric acid 85% (v/v) to pH 2.3. For the preparation of all solutions ultrapure deionized water was used.

2.3 Sample preparation

We analyzed three different partially fermented grapes must from three different manufacture. Samples were filtered through a 0.22 μm syringe filter and diluted to a ratio of 1:5 (v/v) with ultrapure deionized water before direct injection. Aliphatic acids were identified by comparing retention times and spectra with those of standards.

Table 1. Concentration ranges of standard organic acids.

Acid	Concentration range of calibration solutions [mg·L^{-1}]	R^{2*} [%]	RSD** [%]
Oxalic	10.0 - 320.0	0.999	0.302
Tartaric	75.0 - 2000.0	0.999	0.992
Malic	400. - 1600.0	0.999	0.196
Malonic	10.0 - 400.0	0.999	2.862
Lactic	400.0 - 1500.0	0.993	2.329
Acetic	10.0 - 400.0	0.999	2.576
Maleinic	0.5 - 16.0	0.999	0.951
Citric	10.0 - 400.0	0.999	1.262
Succinic	10.0 - 400.0	0.999	2.488
Fumaric	0.5 - 16.0	0.999	0.551
Propionic	10.0 - 400.0	0.999	2.190
Itaconic	10.0 - 320.0	0.999	0.190

*R^2 - correlation coefficient, **RSD – relative standard deviation peak area (n = 5)

3 Results and Discussion

The samples and standard solutions of acids were separated at 25°C by isocratic elution. The chromatograms were acquired at 210 nm. The pH of the mobile phase was chosen to suppress the dissociation of analyzed carboxylic acids. Injected volume of standard solutions mixture was 20 µL and flow rate of mobile phase 1 mL·min^{-1}. Calibrations for the acids were obtained by plotting peak area vs. concentration and the correlation coefficients were in the range 0.993 – 0.999 (Table 1).

In samples of partially fermented grape must we found nine acids: oxalic, tartaric, malic, lactic, citric, acetic, fumaric, maleic and succinic acid (Fig. 1).

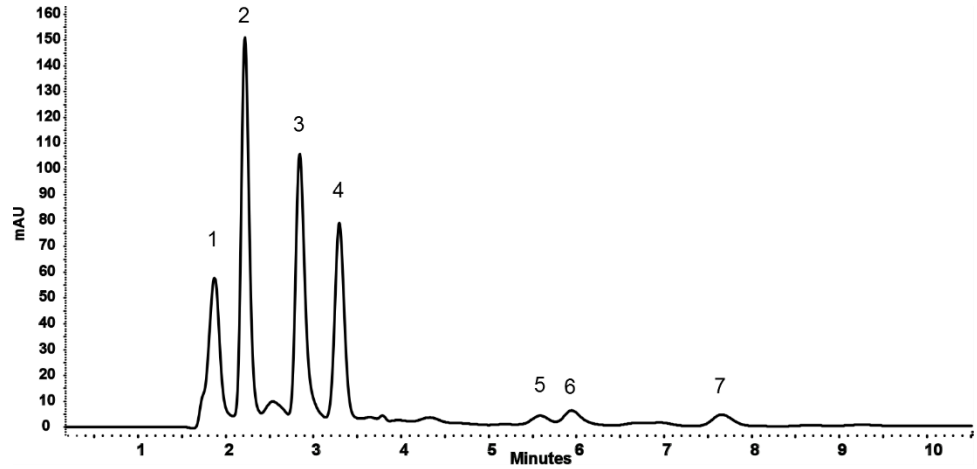

Fig. 1. Chromatogram of partially fermented grape must sample (frist manufacture). Peak identification: (1) oxalic acid, (2) tartaric acid, (3) malic acid, (4) lactic acid, (5) citric acid, (6) succinic acid, (7) fumaric acid.

For each sample, the mean content of the identified analytes is reported in Table 2. For the evaluation of acid samples we used the method of the calibration curve. As we can see, lactic, malic and tartaric acids were the main acids.

Table 2. Mean content (n = 5) of aliphatic acids in three different samples of partially fermented grape must.

Acid	Partially fermented grape must					
	1		2		*3*	
	c [mg·L^{-1}]	*RSD [%]**	c [mg·L^{-1}]	RSD [%]*	c [mg·L^{-1}]	*RSD [%]**
Oxalic	222.04	0.28	548.75	0.43	306.63	0.27
Tartaric	2734.41	0.14	4654.71	0.41	3514.20	0.17
Malic	4402.25	0.31	5267.40	0.38	3830.60	0.56
Lactic	5247.80	0.28	4109.04	0.34	2633.75	0.85
Citric	260.85	3.49	592.31	0.67	356.90	8.05
Succinic	773.42	2.63	765.97	1.63	922.55	8.51
Fumaric	2.45	6.36	9.90	0.74	4.20	10.0
Acetic	-	-	851.25	1.33	691.04	2.55
Maleic	-	-	4.68	1.06	-	-

*RSD – relative standard deviation peak area

4 Conclusions

Ion-exclusion liquid chromatography with spectrophotometric detection at 210 nm is an appropriate method for the determination of organic acids in partially fermented grape must. In this work we propose the use of new type of column for the quantification of twelve aliphatic carboxylic acids.

Partially fermented grape musts from three different manufactures were analyzed. We confirmed the presence of nine acids: oxalic, tartaric, malic, lactic, citric, succinic, fumaric, acetic and maleic acid.

Acknowledgement

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P73 STUDY OF COMPETITIVE BINDING BETWEEN DICLOFENAC AND TRYPTOPHAN ON HUMAN SERUM ALBUMIN USING CAPILLARY ELECTROPHORESIS

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Summary
The capillary electrophoresis fontal analysis (CE-FA) was used to study the affinities between diclofenac and tryptophan, and human serum albumin (HSA). Apparent log K_b = (4.4 ± 0.01) L/mol for diclofenac–HSA and log K_b = (3.95 ± 0.004) L/mol for tryptophan–HSA have a good repeatability. The displacement experiment shows the displacement of tryptophan from binding site using diclofenac.

1 Introduction

Binding to plasma proteins represents one of the basic parameters of every drug. It influences not only the distribution of a drug in the body, but also its availability for biotransformation and thus determines the exposure time of the body to the effect of a drug. Currently, optimizing of all the therapeutic, pharmacokinetic and pharmacodynamic properties plays a key role in the development of new drugs. Binding to plasma protein in body can affect also second drug. For this reason, determination of drug–protein percent of binding in the presence of another drug is important to pharmaceutical applications.

Strength of drug binding to plasma proteins is usually described by binding constant (K_b) value, which can be established using capillary electrophoresis (CE). Different modes of CE can be used to characterize drug–protein interactions for example the capillary electrophoresis fontal analysis (CE-FA), Hummel Dreyer methods, affinity capillary electrophoresis, vacancy peak and vacancy affinity capillary electrophoresis [1].

2 Experimental

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 uncoated fused silica capillary was 58.5/50 cm (L_{tot}/L_{eff}) with I.D. of 75 μm. The BGE was a borate buffer (150 mM sodium tetraborate adjusted to pH 8.5 with 200 mM boric acid). The sample was injected into the capillary with a pressure of 35 mbar at the anode for 40 s. Operational voltage of 14 kV was applied in normal polarity and the detection wavelength was set to 276 nm for diclofenac and 220 nm for tryptophan. The temperature of the capillary was maintained at 25 °C.

3 Results

In order to establish the K_b, CE-FA mode was chosen. Firstly, binding constants (K_b) of simple systems diclofenac-HSA and tryptophan-HSA were determinated. The calculation of K_b from binding curve was performed by nonlinear regression according to formula (1)

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

where: r is the number of drugs bound per protein, n_i, $[D_{free}]$, $[D_{bound}]$ and $[P_{tot}]$ are the maximum number of binding sites on the protein, concentrations of free drug, bound drug and the total concentration of protein, respectively, and K_{bi} is the corresponding binding constant [2-4].

Apparent log K_b values obtained using nonlinear regression were 4.41 ± 0.01 L/mol for diclofenac and 3.95 ± 0.004 L/mol for tryptophan.

Samples in displacement experiments contain diclofenac-tryptophan-HSA. The displacement experiments were performed with various concentration diclofenac in samples. Representative electropherogram obtained with mixture of diclofenac-tryptophan-HSA is shown in Fig. 1.

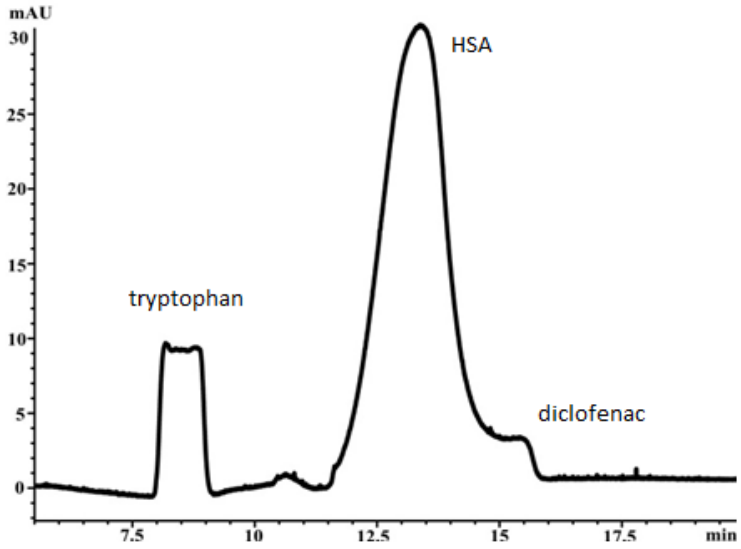

Fig. 1. Representative electropherogram obtained with CE-FA method. Mixture of 100 µM diclofenac, 300 µM tryptophan and 75 µMHSA.

4 Conclusion

The main aim of this work was determination of K_b values for diclofenac–HSA and tryptophan–HSA systems. The subsequent measuring of binding tryptophan to HSA in presence and absent of diclofenac shows displacement tryptophan from binding site. Apparent log K_b values obtained using nonlinear regression was 4.41 ± 0.01 L/mol for diclofenac–HSA and 3.95 ± 0.004 L/mol for tryptophan–HSA.

Acknowledgement
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P74 PERSPECTIVE SWEETENER TAGATOSE AND PH STABILITY

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Summary
Tagatose as a relatively new sweetener has great potential not only in food industry but also in pharmacy. Therefore it is necessary to know all the physical, chemical, microbiological and organoleptic properties.

Stability in pH is very important for the production of food and pharmaceutical products. pH is one of the protective barriers utilized in the manufacture of foodstuffs against microbial infection.

Tagatose stability were screened in the pH range from 2 to 12 and compared with the stability of glucose under the same conditions. The samples were measured by RP-HPLC with RI detection and mobile phase acetonitrile and water and the quantity of stable D-tagatose and D-glucose in samples after 130 days, and compared each other.

1 Introduction
Tagatose is a natural, functional sweetener that has received increasing industry and scientific attention in recent years [1].Tagatose, which is a ketose of galactose, is a sweetener with physicochemical properties similar to sucrose. The structure of tagatose differs from fructose only in the position of the hydroxyl group on the fourth carbon [2].

A small amount of tagatose is found naturally in some dairy foods and fruits. It is used as a sucrose substitute in confectionery, ice cream, soft drinks and cereals [3].

Tagatose provides several health beneficial effects, including the promotion of weight loss, no glycemic effect, anti-plaque, non-carcinogenic and prebiotic properties. Prebiotic property of tagatose is the most challenging to the food industry. Only minimal amount of tagatose is absorbed in the upper gastrointestinal tract and the rest, unabsorbed of tagatose is fermented in the intestines to yield short chain fatty acids, including propionic and butyric acids. Tagatose consumption generated a greater number of lactic acids bacteria while decreasing the number of coliform bacteria [3].

Tagatose is commercially produced from lactose through a combination of chemical and enzymatic processes. It was classified as generally recognized as safe (GRAS) by the U. S. Food and Drug Administration (FDA) in 2001 [1].

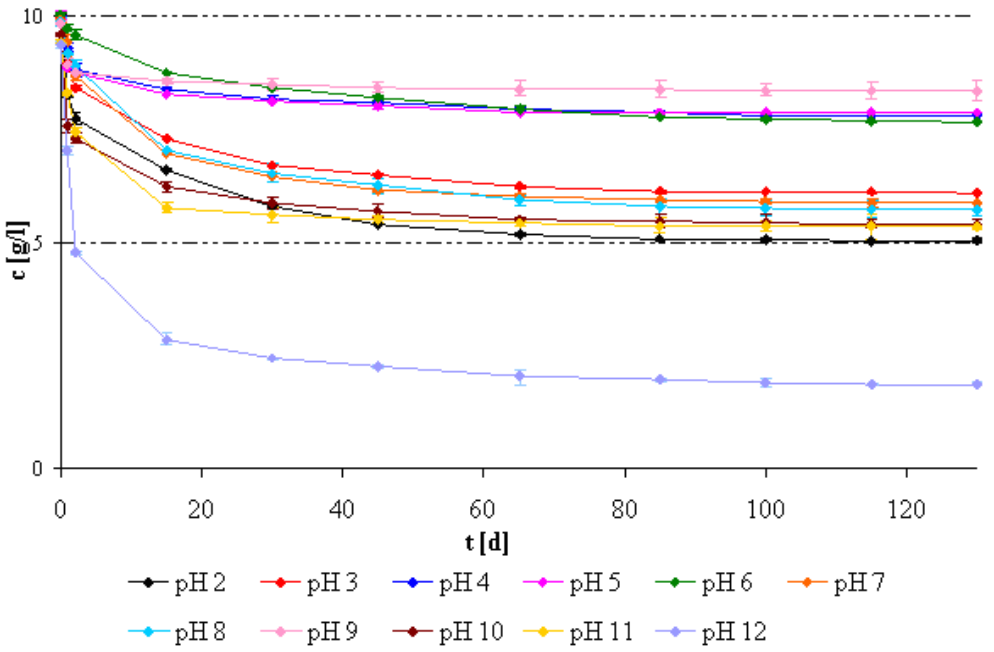
2 Experimental

Two aqueous stock solutions of D-tagatose, Sigma-Aldrich and D-glucose, Lachema were prepared of the exact concentration 10 $g.l^{-1}$. From these solutions were prepared calibration ranges, which were used for the actual determination. Thereafter, 2 ml of stock solutions were pipetted into vials and HCl and NaOH were added to a final pH 2–12. Thus prepared solution with a given pH was measured by HPLC and taken as the sampling time 0^{th}.

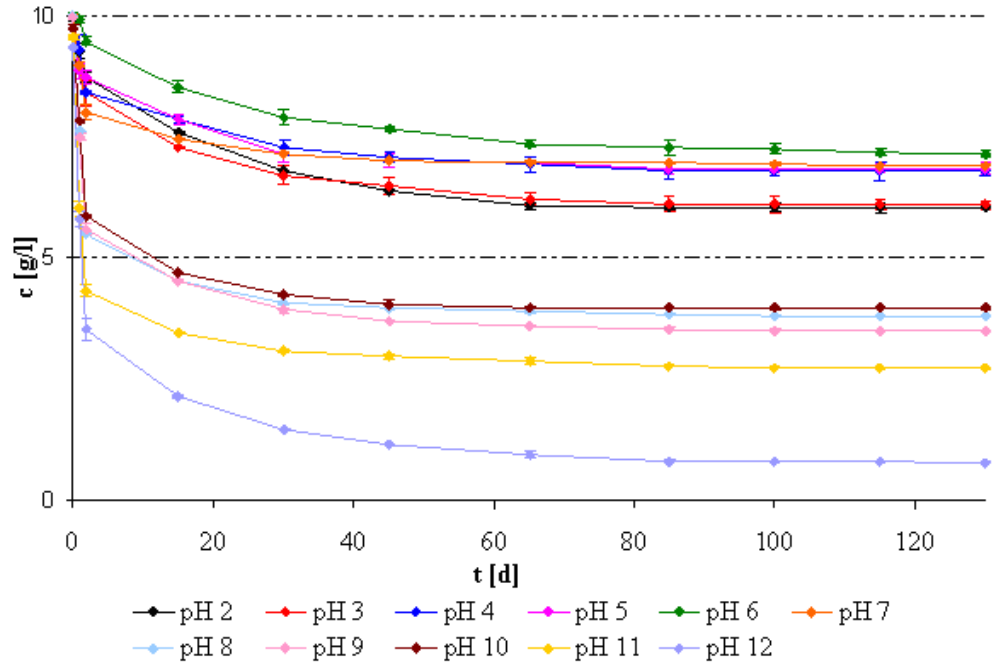
Subsequently, sample were performed in time interval and measured on a Shimadzu HPLC assembly 10A with RID and column Prevail™, Grace Davidson Discovery Science 250x4,6 mm, 5 μm under condition of mobile phase 75:25 ACN:H_2O and 40 °C and mobile phase flow rate of 1,5 $ml.min^{-1}$.

3 Results and Discussion

From the graph of degradation D-tagatose (Graph 1) we can say that all samples, without pH 12, are report more than 50 % stability of aqueous solution D-tagatose after 130 days. Unlike D-glucose, from graph degradation D-glucose (Graph 2) we can say that aqueous solution with pH higher than 7 are not stable after 130 days, because their percentage of stability is lower than 50 %, Table 1.



Graph 1. Degradation D-tagatose at various pH in the time interval.



Graph 2. Degradation D-glucose at various pH in the time interval.

The obtained results we can confirm the literature that says that D-tagatose is stable in the pH 2 to 7 [4]. This range adjusted to the area with higher stability (pH 4 to 6) and lower stability (pH 2 to 4 and 7 to 11).

Table 1. Stability of D-tagatose and D-glucose after 130 days storage in pH [%].

pH	*D-tagatose*	*D-glucose*
2	50,3 ± 0,4	60,3 ± 0,1
3	60,9 ± 0,0	60,9 ± 1,0
4	77,8 ± 0,0	67,8 ± 1,1
5	78,3 ± 0,1	68,4 ± 1,1
6	76,4 ± 0,1	71,5 ± 0,6
7	58,6 ± 0,1	68,8 ± 0,4
8	57,1 ± 0,7	37,9 ± 0,0
9	83,2 ± 1,1	34,9 ± 0,0
10	53,9 ± 0,1	39,5 ± 0,1
11	53,3 ± 0,6	27,1 ± 0,1
12	18,6 ± 0,5	7,7 ± 0,2

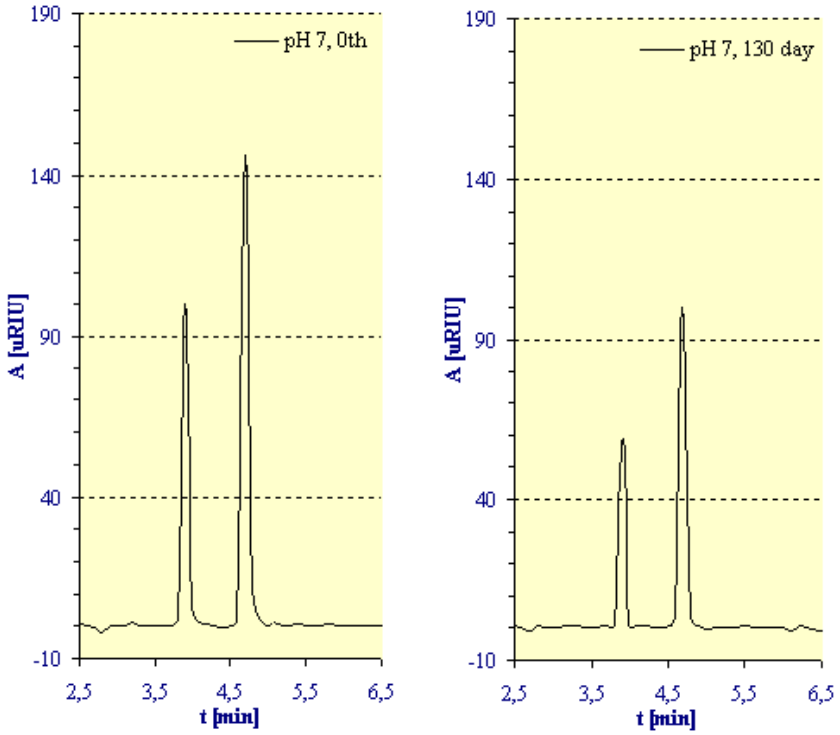


Fig. 1. Example of chromatogram at pH 7 at time 0th and 130 days, first peak is D-tagatose and the second is D-glucose.

4 Conclusions

From the results obtained by measurement on HPLC can be said that samples of D-tagatose exhibit generally higher stability than samples with D-glucose. 10 samples of D-tagatose have stability higher than 50 % after 130 days of monitoring. Unlike glucose, only 6 samples have stability higher than 50 %. The decrease of D-tagatose is milder than D-glucose. Steeper decline in D-tagatose was conducted in 30 day, while glucose lasted up to 65 days.

Tagatose has greater potential for long-term storage in acidic and mild alkaline condition. And it is also suitable for processing and storage of alkaline food types.

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P75 DIRECT COUPLING OF SUPPORTED LIQUID MEMBRANE EXTRACTIONS TO CAPILLARY ELECTROPHORESIS. SENSITIVITY ENHANCEMENT IN ANALYSES OF UNTREATED COMPLEX SAMPLES

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Summary

Extractions across supported liquid membrane (SLM) were in-line coupled to capillary electrophoresis (CE) commercial system for direct injection of basic drugs from raw complex samples. A micro-extraction unit was inserted into the CE system sampling device and ensured excellent sample clean-up and injections directly from the membrane surface. Transient isotachophoresis (t-ITP) combined with large electrokinetic injections of selectively extracted basic analytes increased the sensitivity of the analytical method up to 300-fold when compared with conventional hydrodynamic injections and capillary zone electrophoresis (CZE) of the basic analytes.

1 Introduction

Analysis of complex samples is not a trivial task owing to the fact that their matrix usually contains large number of compounds, which are often present in high concentrations, and interfere with the determination of analytes. Moreover, complex samples often contain high-molecular mass matrix components, which result into analytical instrumentation fouling. Efficient matrix removal is therefore necessary prior to vast majority of analytical procedures. This is mostly performed in an off-line fashion using large volumes of complex samples and reagents, is costly and time consuming. An effective alternative to off-line approaches is direct coupling of miniaturized sample treatment to analytical methods, which may eliminate the need for user intervention, reduce the sample consumption, costs and environmental impact. SLMs were shown suitable for direct coupling to CE enabling efficient sample clean-up of complex samples, nevertheless, preconcentration was not possible in the reported set-up [1, 2]. In this contribution, a simple approach is described,

which significantly enhances sensitivity of the SLM-CE method, while keeping its advantages, i.e. automation, minimum sample volumes, low costs and high speed.

2 Experimental

2.1 Capillary electrophoresis

A 7100 CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with UV-Vis absorbance detector (direct detection at 200 nm) was operated at a potential of + 15 kV applied at the injection side of the separation capillary and was maintained at 25°C. BGE solution consisted of 5 M acetic acid. Hydrodynamic injections were performed at 30 mbar for 5 s. 300 mM ammonium acetate was injected at 35 mbar for 25 s prior to the sample injection and was used as the t-ITP plug for stacking of basic analytes. Extracted analytes were injected electrokinetically from the SLM surface at + 5 kV for 60 s just behind the t-ITP plug.

2.2 Coupling of SLMs to CE

The basic concept for coupling extractions across SLMs to CE was described previously [1, 2]. Tailor-made thin-wire springs were used for accommodation of the micro-extraction units with SLMs into Agilent standard plastic vials. 20 µL of raw biological sample and 10 µL of 10 mM acetic acid were pipetted as donor and acceptor solution into the respective part of the unit. 1-ethyl-2-nitrobenzene was used as liquid membrane. Extraction time was set at 20 min.

3 Results and Discussion

In order to achieve efficient preconcentration of slow cationic analytes a long plug of rapidly migrating cation is injected into separation capillary, which forms a zone with sharp rear boundary, first. Second, analytes are injected behind this plug and are stacked at the boundary during a CZE run. Using this so called t-ITP process, large quantities of analytes can be injected behind the plug since the stacking process ensures good separation efficiency and a significant increase of sensitivity is reached. Ammonium was selected as a suitable cation for the t-ITP preconcentration step. The following procedure was applied: (i) the SLM extraction unit was filled with respective solutions and placed into the plastic vial for extraction, (ii) ammonium acetate was hydrodynamically injected and (iii) drugs extracted across the SLM were electrokinetically injected into the separation capillary. High voltage was then applied to the CE system and the t-ITP-CZE method was initiated. Improvement of the SLM-t-ITP-CZE method sensitivity compared to conventional SLM-CZE with hydrodynamic injection was between 50 and 300 for the three drugs, as can be seen in Figure 1. The method was also applied to direct injection and preconcentration of basic drugs from untreated human urine and human serum samples.

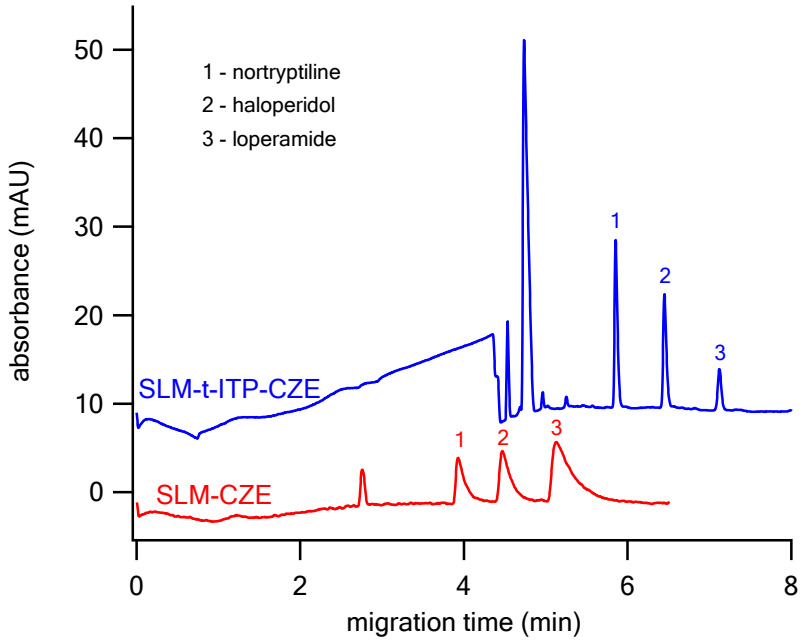

Fig. 1. Comparison of SLM-CZE and SLM-t-ITP-CZE method sensitivity for determination of basic drugs in standard solutions. SLM, CZE and t-ITP-CZE conditions, see Section 2. Concentrations of drugs, 30 mg/L (SLM-CZE) and 0.5 mg/L (SLM-t-ITP-CZE).

4 Conclusions

A simple method that combines t-ITP-CZE with electrokinetic injection of basic drugs extracted and directly injected from SLMs was presented. The method offers excellent sensitivity and minimizes sample handling to solely pipetting of less than a drop of a complex sample into disposable extraction unit. All other procedures, such as extraction, injection, separation and quantification, are performed in a fully automated manner. Additional advantages of the method are minimum consumption of organic solvents, rapid extractions/analyses and applicability to raw, undiluted complex samples.

Acknowledgement

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

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P76 DNA POLYMERASE STOP ASSAY FOR DETECTION OF G-QUADRUPLEXES

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Summary
In this work we performed DNA polymerase stop assays while using template DNA derived from promoter regions of *VEGF* and *c-Myc* proto-oncogenes under variety of experimental conditions. Partial stopping of DNA synthesis along the template strand was observed in the presence of K^+ ions due to formation of stable G-quadruplex structures. In contrast, Na^+ ions alone were unable to stabilize G-quadruplexes to stop the reaction at their site. These data suggest that K^+ and Na^+ ions, which are both known to stabilize G-quadruplexes, do this in different manner or extent.

1 Introduction

Four-stranded DNA consisting of stacked guanine quartets, so-called G-quadruplex or G4-DNA, has been proposed to function in several biological processes including DNA replication and gene transcription. Guanine-rich DNA strands capable of adopting intramolecular G-quadruplex structure *in vitro* are frequently derived from telomeric or gene regulatory regions. G-quadruplex motifs have been identified in promoters of proto-oncogenes including *c-Myc*, *c-Kit*, *hTERT*, *KRAS* or *VEGF* [1]. Recently, G-quadruplex DNA has been observed to form in genomic DNA of human cells by using structure-specific antibody [2].

DNA polymerase stop assay belongs among methods which are used to study G-quadruplex formation *in vitro* [3]. DNA polymerase enzyme is used to extend DNA primer on DNA template that includes G-quadruplex forming sequence. Upon formation of stable G-quadruplex in suitable conditions, DNA polymerase stops synthesizing complementary DNA strand at the site of its occurrence. In this work DNA polymerase stop assays with labelled primers were performed, as templates were used G-rich strands from promoter regions of *c-Myc* and *VEGF* genes.

2 Experimental

DNA polymerase stop assay was performed with radioactively labelled primer PS28. Oligonucleotides Pu77-PS, VEGF-PS and A50-PS were used as template DNA strands. Sequences are presented in Figure 1. Polymerase reactions were performed in the presence 0 – 100 mM of KCl and NaCl. Various DNA polymerases were used: KOD XL (KOD XL DNA polymerase, Novagen), KLENOW1 (Large Fragment from DNA polymerase I, NEB) and KLENOW2 (Large Fragment from DNA polymerase I,

Fermentas). Electrophoresis was performed on 16 % denaturing PAGE gel. Schematic representation of primer extension products is shown on Figure 1.

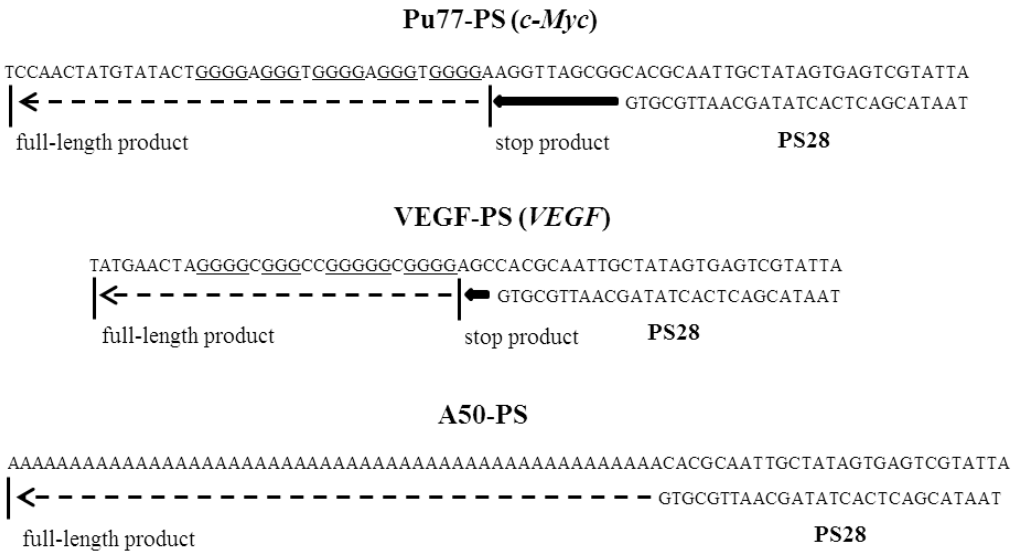

Fig. 1. Oligonucleotides used in DNA polymerase stop assay. Full-length products and stop products at the site of G-quadruplex formation are indicated. Primer PS28 was extended along various template strands: Pu77-PS (*c-Myc* gene), VEGF-PS (*VEGF* gene) and A50-PS that was used as control.

3 Results and Discussion

DNA polymerase stop assay was used to detect G-quadruplex formation on template DNA under favorable conditions. Figure 2 shows DNA polymerase products of Pu77-PS, VEGF-PS and A50-PS which were incubated with rising concentrations of K^+ ions. DNA synthesis on Pu77-PS and VEGF-PS templates was partially stopped at G-quadruplex motif upon addition of K^+ ions in the buffer. Ion composition had no effect on A50-PS template polymerase reaction as only full-length product is visible. These data support that K^+ ions stabilize G-quadruplexes in those regions and DNA polymerase moving along template strand is therefore blocked. However, in the presence of Na^+ ions, DNA polymerase was able to synthesize the complementary strand as G-quadruplex was either not formed or was not stable enough to block the enzyme.

Next, we examined if temperature or DNA polymerase used had any effect on polymerase reaction. Figure 3 shows products of DNA polymerase reaction on VEGF-PS template DNA. Stop products were present in reaction with all used polymerases and temperatures in the presence of 100 mM KCl which again supports stabilization of G-quadruplex structure on VEGF-PS.

G-quadruplexes are stabilized by both K^+ and Na^+ ions, which was demonstrated by CD spectroscopy on thrombin binding aptamer and various telomere-derived sequences [4]. Stronger stabilization by K^+ ions is in accordance with our results that polymerase stop product appears in reactions supplemented with K^+ ions.

Elevated occurrence of G-quadruplex motifs in gene promoter regions implies their possible role in regulation of gene expression [5]. Both *c-Myc* and *VEGF* genes are coding proteins that are important in cancer development and formation of G-quadruplexes in their regulatory regions could present a role for G-quadruplex *in vivo* function. Structure-specific interaction of DNA-binding proteins may yield a step in regulation of those and others genes that are frequently overexpressed in cancer cells. Small molecules functioning as G-quadruplex-binding ligands are also being developed with perspective that they could serve as therapeutic agents. DNA polymerase stop assay is a method that can help in analyzing impact of various ligands and proteins on G-quadruplex DNA [6].

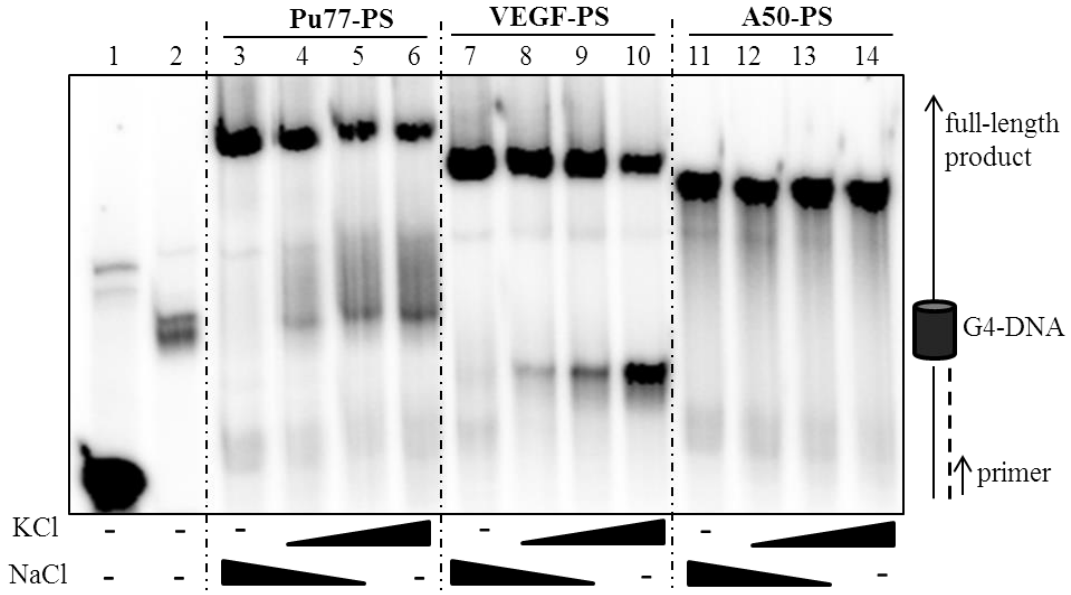

Fig. 2. DNA polymerase stop assay performed under various K^+ and Na^+ concentrations. Template DNA was incubated without or with 50 mM, 75 mM or 100 mM KCl prior to polymerase reaction. Concentration of K^+ ions was compensated with Na^+ ions. KLENOW2 was used for this experiment as DNA polymerase.

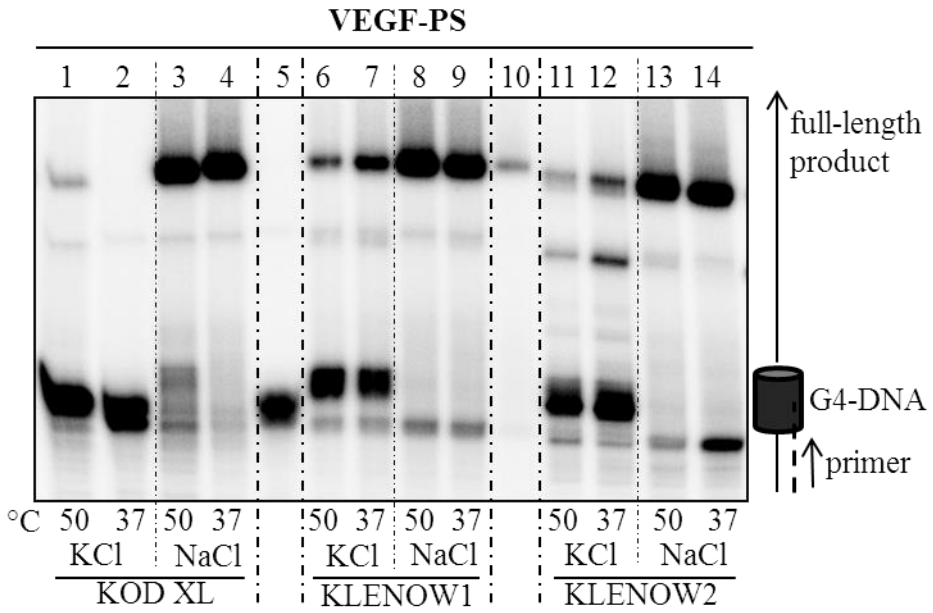

Fig. 3. DNA polymerase stop assay performed with various DNA polymerases (KOD XL, KLENOW1 and KLENOW2). Reactions were performed in either 100 mM KCl or 100 mM NaCl at temperatures 37 ºC or 50 ºC. VEGF-PS oligonucleotide was used as template.

4 Conclusions

In this work we used DNA polymerase stop assay to detect G-quadruplex DNA structure formed in DNA designed from promoter regions of *c-Myc* and *VEGF* genes. Our data show that these G-quadruplexes are stabilized by K^+ ions and are stable enough to obstruct DNA polymerase and stop DNA synthesis along template strand. DNA polymerase stop assay can be further used to study various molecules including proteins that interact with G-quadruplexes such as mutant p53 proteins [7]. It may represent a tool for clarifying their effect on G-quadruplex formation and stability.

Acknowledgement

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P77 GENETIC DIVERSITY OF CZECHOSLOVAK ORIGIN RYE CULTIVARS DETECTED BY RAPD MARKERS

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1 Introduction

Rye (*Secale cereale* L.) is mainly a European cereal with about 75 % of the global production growing in Russia, Belarus, Poland, Germany and Ukraine. It has the best overwintering ability, and the highest tolerance to drought, salt, or aluminium stress from all small-grain cereals. Harvest is used for bread making, feed, and in growing demands for ethanol and biomethane production as a renewable energy source [1].

Molecular markers are used to determine genetic similarity by scientists in different fields. The use of molecular markers such as RAPD offers a way to better characterize and describe plant species. It can determine the range of genetic similarity between species, explicitly indicating the possibility of crossing between genotypes [2, 3]. The aim of this study was to assess genetic similarity of the Czechoslovak origin rye genotypes using 3 RAPD primers.

2 Experimental

2.1 Plant materials

In total, seeds of sixteen winter rye cultivars (*Secale cereale* L.) (Table 1) were used in RAPD analyses. Samples of rye were obtained from the Gene Bank of the Slovak Republic of the Plant Production Research Center in Piešťany. Fifteen genotypes of rye came from Czechoslovakia and one genotype of rye came from Czech Republic.

2.2 Genomic DNA isolation

DNA was isolated from the 14 days leaves with GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Scietific, Gdańsk, Poland) according to the manufacturer's instructions. DNA concentrations were estimated by UV-Vis

spectrophotometer Q5000, Quawell. RAPD analysis was performed using eight 10-mer random primers (Table 2) procured from Genomed, Warsaw, Poland.

Table 1. List of used genotypes of rye, their Plant species and country of origin.

Genotype	Plant species	Country of origin
Valtické	S. cereale L. subsp. cereale var. cereale	CSK
Tešovské	S. cereale L. subsp. cereale var. cereale	CSK
Keřkovské	S. cereale L. subsp. cereale var. cereale	CSK
Zenit	S. cereale L. subsp. cereale var. cereale	CSK
Chlumecké	S. cereale L. subsp. cereale var. cereale	CSK
České	S. cereale L. subsp. cereale var. cereale	CSK
Albedo	S. cereale L. subsp. cereale var. cereale	CSK
Židlochovický Panis	S. cereale L. subsp. cereale var. cereale	CSK
Nalžovské	S. cereale L. subsp. cereale var. cereale	CSK
Dobrovické	S. cereale L. subsp. cereale var. cereale	CSK
Vigľašské	S. cereale L. subsp. cereale var. cereale	CSK
Ratbořské	S. cereale L. subsp. cereale var. cereale	CSK
Laznické	S. cereale L. subsp. cereale var. cereale	CSK
Breno	S. cereale L. subsp. cereale var. cereale	CSK
Dobřenicke krmné	S. cereale L. var. Multicaule	CSK
Aventino	S. cereale L.	CZE

2.3 RAPD-PCR analysis

Polymerase chain reactions (PCR) were carried out in 25 µl of following mixture: 10.25 µl deionized water, 12.5 µl Master Mix (2x Master Mix, A&A Biotechnology, Gdynia, Poland), 1.25 µl of genomic DNA, 1 µl of primer. PCR amplifications were performed on a labcycler (Sencoquest, Göttingen, Germany) following amplification profile: an initial denaturation step at 94 °C for 1 min, followed by 10 cycles of amplification 5s at 94 °C, 30 s at 37 °C and 30 s at 72 °C and next 35 cycles of 5 s at 94 °C, 30 s at 37 °C and 1 min at 72 °C. Amplified products were analyzed by gel electrophoresis in 1 % agarose gel at 170 V for 1.5 h. The gels were visualized by Midori Green staining (Nippon Genetics Europe GmbH, Düren, Germany).

2.4 Data analysis

RAPD bands were scored as 1 for presence and 0 for absence. Consequently, using binary matice a dendrogram based on hierarchical cluster analysis using UPGMA (Unweighted Pair Group Method using arithmetic Averages) algorithm with the SPSS professional statistics version 17 software, package was constructed.

Table 2. List of RAPD primers with their Sequence and Chromosomal location.

Primer´s name	Sequence	Chromosomal location
RLZ 2	5´AGAGATCTCC 3´	5RL
RLZ 3	5´TGTCCAGCTT 3´	2RL
RLZ 4	5´TCGCCCCATT 3´	2RS

3 Results and Discussion

The use of molecular markers (RAPD) are routine methods for quick and efficient relationships estimating between lines and populations of many plant species [4].

For the analyse of 16 rye species of Czechoslovak origin we used three RAPD primers by those we detected 24 DNA fragments at 3 loci with an average number of 8 fragments per locus. From the detected DNA fragments, 21 (84,9%) were polymorphic, which is relatively high number for rye genotype differentiation of analyzed collection. Reference [5] used 15 RAPD markers for detection genetic relationship between ten genotypes of rye. The number of fragments was in the range 5 -21 with an average 9,93. Percent of polymorphism was from 50,0 % to 83,3 % with an average 68,9 %.

For determination of the genetic relationships between rye genotypes a dendrogram was used. The dendrogram was constructed based on principle of hierarchical cluster analysis using UPGMA algorithm in statistical program SPSS. Analyzed rye genotypes were divided into three major clusters, where the first contained 11 genotypes of Czech and Czechoslovak origin, the second one single genotype (Chlumecké) and the third cluster included 4 rye genotypes of Czechoslovak origin. In the dendrogram it was unable to distinguish two rye genotypes (Víglašské and Aventino) (Fig.1). Only three RAPD primers used were able to distinguish almost all rye genotypes, what indicates their good differentiation ability. Division of genotypes in to clusters may be caused by differences in genetic background. The results showed that RAPD markers are useful for exploring germplasm diversity which is raw material for developing new varieties [3]. Reference [6] shows dendrogram constructed based on RAPD markers using UPGMA algorithm that divided 42 genotypes of rye into two main clustres: spring and winter group.

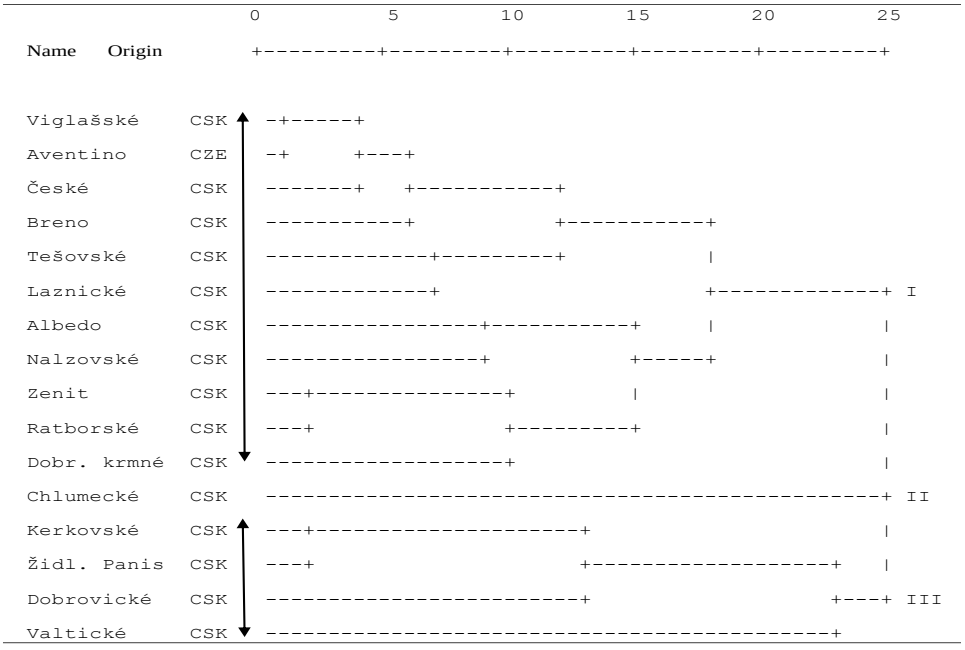

Fig. 1. Genetic similarity dendrogram of rye genotypes, from RAPD marker analysis.

A UPGMA-dendrogram within the winter and spring groups showed that the clusters corresponded well to their geographical locations. RAPD markers have been used in many studies for DNA fingerprinting and phylogenetic analyses [3, 7].

4 Conclusions

RAPD markers were used as a powerful tool for assessment of genetic diversity in rye cultivars. The results showed that genotypes of rye were divided into three major clusters. We could not distinguish two genotypes (Viglašské a Aventino). For distinguishing of all rye genotypes it is necessary to use more random RAPD primers.

Acknowledgement

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P78 THE WAYS OF ACTIVATING TP53

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1 Introduction

The tumor suppressor p53 protein (wtp53) is multifunctional protein; one of its most important functions is transactivation or transrepression of about 200 target genes. It regulates cellular response to DNA damage, caused by different types of stress (for example UV radiation, ionizing radiation, reactive oxygen species and anticancer drugs) [1]. DNA damage induced by ROS involves base modification, apurinic sites and DNA strand breaks. A frequent modification of DNA is modified guanine (8-oxo-7,8-dihydro-2'-deoxyguanosine), that is routinely used as a measure of oxidative stress [2]. Protein p53 is stabilized and activated by phosphorylation and acetylation under stress conditions. After these events, p53 activates its target genes through sequence specific interactions with DNA sequences (p53CON) in gene promoters responsible for regulating of cell cycle, inducing apoptosis and DNA repair. p53 is central molecule in maintaining genomic integrity, thus mutation in *TP53* gene occurs in about 50 % of cancer cases. Expression of mutant p53 protein is associated with increased treatment resistance of cancer cells, inability to regulate standard target genes and gain of other oncogenic functions. Zinc ion is an important cofactor in p53 structure, because it stabilizes the tertiary structure. Many p53 mutant proteins have destabilized protein conformation and lose their zinc ion [2, 3]. In our work we showed, that adding zinc supports the effect of cytostatics on cancer cell lines with different p53 status.

In our work we compared effect of 5-fluoruracil with other cytostatic drugs (doxorubicin, etoposide and cisplatin) mainly on colon cancer cell lines HCT116 with different p53 status. 5-fluoruracil is still the most effective adjuvant cytostatic for patients with colon cancer. Main mechanism of its function is inhibition of DNA and RNA synthesis. Doxorubicin is well known for intercalation to DNA, etoposide belongs to the topoisomerase inhibitors and finally cisplatin causes crosslinking of DNA, which triggers apoptosis; but mechanisms of its action are wider.

In our work we focused on different ways of p53 activation in HCT colon cancer cell lines containing wtp53 and mutant p53 proteins [1] and on the role of zinc supplementation for activating p53.

2 Experimental

2.1 Cell lines and MTT assay

Human colorectal adenocarcinoma cell lines HCT116 with wild type p53 $^{(+/+)}$ and its derivates with deleted *TP53* $^{(-/-)}$ and mutant *TP53* $^{(R248W/-)}$ were provided by Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD, USA) [4]. HCT116 cells were maintained in DMEM (Hyclone, Gaithersburg, MD, USA) with 5 % FBS (fetal bovine serum) and antibiotics penicillin and streptomycin.

The cytotoxic effect of doxorubicin and its combination with zinc supplementation on HCT116 cells was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, HCT116 cells ($7{,}5x10^3$/well) were incubated with a serial dilution of doxorubicin in 96-well plates. At 24 h or 48 h after incubation, 20 µl of MTT solution was added to each well and incubated for an additional 4 h. The colored formazan crystal produced from MTT was dissolved in MTT solvent (4 mM HCl, 0,1% NP-40 in isopropanol) and OD values of the solutions were measured at 595 nm by a plate reader.

2.2 Immunoblot analysis of proteins

Cells were treated with cisplatin (1 µM), etoposide (10 µM), doxorubicin (0,1 µM) or 5´- fluorouracil (5 µM) for 24 hours. In case of pretreatment with zinc they were supplemented by $ZnCl_2$ (100 µM) as well for 24 hours.

An ECL Western Blotting Detection Reagents was purchased from Amersham, GE Healthcare and BioTrace nitrocelulose membrane was obtained from PALL, Life Sciences. The anti-p53 (DO-1) antibody, anti-p21, anti-MDM2 and anti-PCNA were purchased from Sigma Aldrich.

2.3 qRT-PCR of *TP53* and its target genes

Cells were treated with cisplatin (1 µM), doxorubicin (0,1 µM) or 5´- fluorouracil (5 µM) 24 hours before mRNA purification (NucleoSpin RNA II. kit, MachereyNagel). For qRT-PCR analysis, 2 µg of RNA were reverse transcribed by the High Capacity RT kit (Applied Biosystems). PCR was performed using the EvaGreen (Solis Biodyne) in the standard program running in a RotorGene 6000 (Corbett Research). PCR reactions for each sample were done in triplicates. The housekeeping gene (GAPDH) was used as endogenous control. Relative quantitation of transcript levels with respect to the calibrator was done based on $R = 2^{-\Delta\Delta Ct}$ algorithm.

3 Results and Discussion

3.1 Comparison of cytostatic effect of cisplatin, doxorubicin and 5-FU on HCT116 wt ($p53^{+/+}$) cells

To examine p53 activation in HCT116 $p53^{+/+}$ cells by cisplatin, 5-fluorouracil and doxorubicin cytostatics; *p21*, *BAX* and *TP53* gene expression was measured by qRT-PCR (Fig.1A) after 20 hours of treatment. Activation of p53 target genes was compared with activation on protein level (Fig.1B). We observed significant induction of *TP53*, *p21* and *BAX* gene expression by 5-fluorouracil (20 hours treatment); *p21* and *BAX* by cisplatin and *p21* and *TP53* by doxorubicin.

Similar observation we received on protein level, where we treated HCT116 $p53^{+/+}$ cells by cytostatics. We observed significant induction of proteins such as p53, p21 and MDM2 after cytostatic treatment. Proteins p53 and p21 were activated by doxorubicin, etoposide and 5-fluorouracil, while MDM2 was activated only by doxorubicin and 5-fluorouracil. The cisplatin was the least effective, as seen in Fig.1 in qRT-PCR as well as from protein level. The results with 5-fluorouracil confirm previous observations [1].

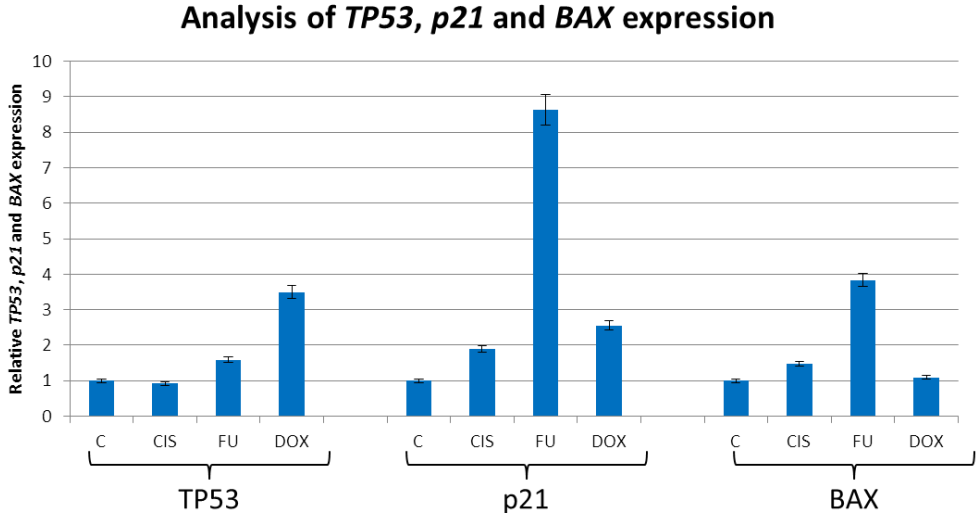

Fig. 1A. *TP53*, *p21* and *BAX* were upregulated in HCT116 $p53^{+/+}$ cells following DNA damage by cytostatic stress by cisplatin, 5-fluorouracil and doxorubicin.

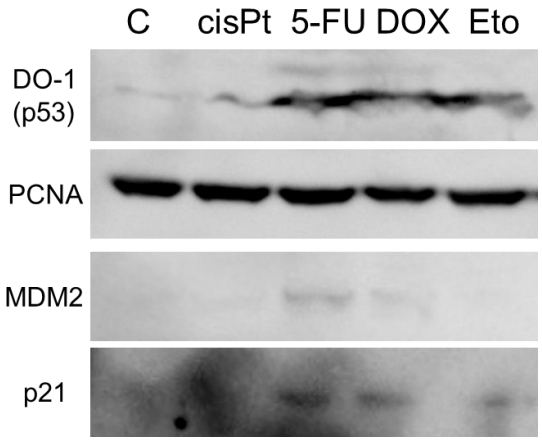

Fig. 1B. Western blot of p53, PCNA, MDM2 and p21 expression in HCT116 $p53^{+/+}$ cell lines.

3.2 Zinc improves drug-induced cell death in mutant p53 and wild-type expressing cells

Cytotoxicity of doxorubicin, cisplatin and 5-FU was investigated in HCT116 $p53^{-/-}$, HCT116 $p53^{+/+}$, HCT116 $p53^{R248W/-}$ cells by several cytotoxicity assays (MTT, WST-1, trypan blue exclusion). To evaluate whether zinc could influence the effect of cytostatic on HCT116 cells**,** viability of HCT116 cells after cytostatic treatment was

compared with cells supplemented by zinc 24 hours before treatment. We observed significant enhancement of cell death in both (wtp53 and mtp53) cell lines after zinc supplementation prior to doxorubicin treatment as shown on the examples of MTT assays (Fig.2). Effect of combining 24 hours dose of doxorubicin with zinc supplementation was comparable to the effect of 48 hours treatment of doxorubicin alone [4].

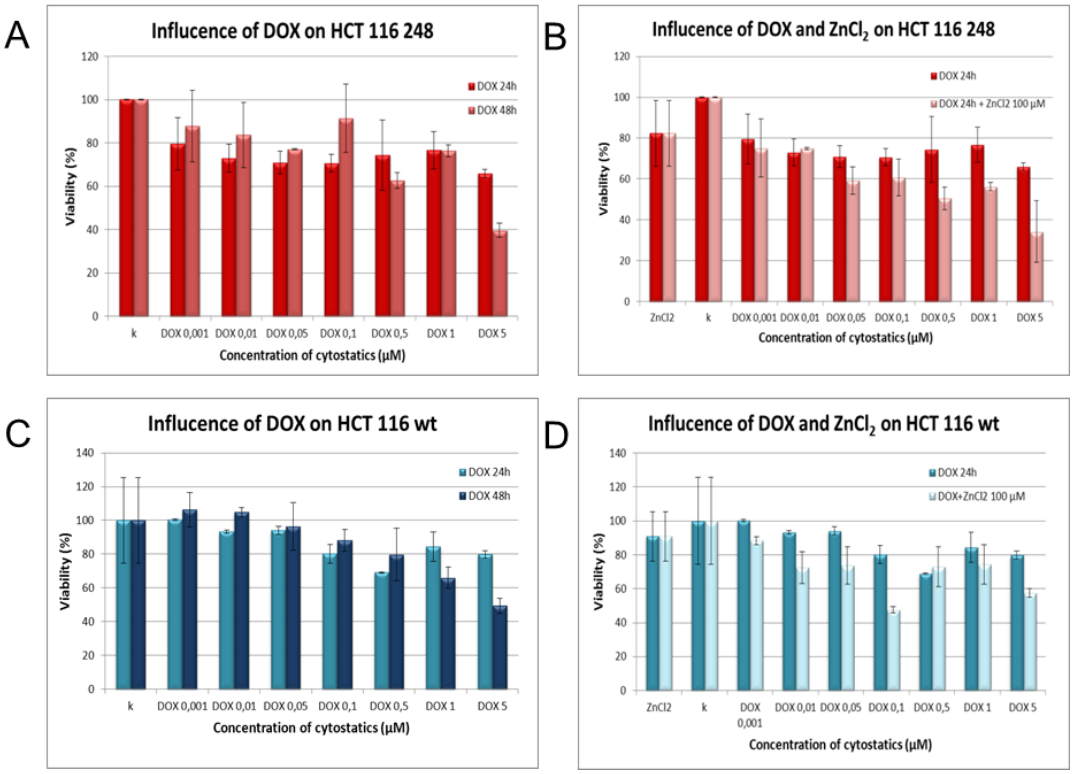

Fig. 2. MTT assay. A,C comparison between doxorubicin treatment for 24 respectively 48 hours. B,D influence of zinc supplementation.

4 Conclusions

We investigated ways of activation of *TP53* under stress conditions induced by cytostatic treatment in colon cancer cell lines with different p53 status. qRT-PCR showed that 5´-fluorouracil and doxorubicin gave the best results in the activation of the target gene. Our results also showed that zinc supplementation might have positive effect on anti-tumor drug treatment of cell line HCT116 $p53^{R248W/-}$ expressing mutant p53 as well as wild type p53 expressing cell line HCT116 $p53^{+/+}$.

Acknowledgement

The research was supported by GACR (13-36108S), IGA VFU Brno 103/2013/FaF and IGA VFU Brno 67/2014/FaF.

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P79 PLASMA FREE METANEPHRINES IN DIAGNOSTICS

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Summary
Quantitative determination of catecholamines and their O-methyl metabolites (metanephrines) plays an important role in the diagnosis of pheochromocytoma (PHEO) – adrenal medulla tumour. The most common symptom of this kind of tumour is hypertension (high blood pressure). PHEO synthesizes, stocks, metabolizes and mostly secretes catecholamines. For this reason it is possible to use significantly elevated concentrations of catecholamines and their metabolic products as diagnostic markers of this tumour. The determination of metanephrines, mainly normetanephrine (NMN), metanephrine (MN) and 3-methoxytyramine (3-MT), is preferred against the determination of catecholamines because tumour cells produce free metanephrines continuously and irrespective of the release of catecholamines.

The project aims to develop a new kit for the determination of plasma metanephrines. Solid phase extraction (SPE) is used for the pre-treatment of plasma samples.The determination is performed by ion-pair ultra-high performance liquid chromatography with electrochemical detection (UHPLC-ED).

1 Introduction

Catecholamines are organic compounds derived from the amino acid tyrosine [1]. Tyrosine is produced in the human body by hydroxylation of phenylalanine [2]. Catecholamines act as hormones or neurotransmitters. These substances are produced by the central nervous system or the adrenal medulla. In a healthy human body there are these compounds and their metabolites represented in very small amounts (pmol/L) [3, 4]. Higher levels of catecholamines and their metabolites are in the body

caused by stress, physical exertion, pain, emotional distress, etc. Extremely high levels of catecholamines and metabolites are caused by neuroendocrine tumours of the adrenal medulla, which include e.g. pheochromocytoma [5].

PHEO synthesizes, stocks, metabolizes and mostly secretes catecholamines. These compounds and their metabolic products are used as diagnostic markers of the mentioned tumour [4]. The determination of metanephrines, particularly metanephrine, normetanephrine and 3-methoxytyramine, is often preferred against the determination of catecholamines regarding the diagnosis of pheochromocytoma. Tumour cells produce free metanephrines continuously and irrespective of the release of catecholamines [5, 6]. Moreover 3-MT is a biomarker for metastatic PHEO. [7].

2 Experimental

2.1 Instrumentation

The chromatographic system consists of the Ultimate 3000 Series pump, the Ultimate 3000 Series ACC-3000 autosampler (both from Thermo Fisher Scientific, Inc., Waltham, USA) and a core-shell column. It is used the Coulochem III detector containing one conditioning cell (Model 5021A) and one analytical cell (Model 5011A) from ESA, Inc., Chelmsford, USA.

2.2 Methods

2.2.1 Pre-treatment of human plasma sample

Solid phase extraction with ion exchange columns is used for a pre-treatment of blood plasma samples. The eluate is evaporated and the residue is dissolved in 220 µL of mobile phase, of which 150 µL is injected onto the chromatographic column.

2.2.2 UHPLC/Coulochem analysis

The determination of MN, NMN and 3-MT is performed by ion-pair ultra-high performance liquid chromatography (UHPLC) with electrochemical Coulochem III detector. 4-hydroxy-3-methoxybenzylamine (HMBA) is used as an internal standard. The column is heated to 28°C, the autosampler temperature is set at 8.5°C and a flow rate of mobile phase at 0.5 mL/min. The potential of conditioning cell is set at +400 mV. The working potentials are +100 mV (1st electrode) and -350 mV (2nd electrode).

3 Results and Discussion

During the development of the kit we have progressively optimized the whole method. Pre-treatment of plasma sample, solid phase extraction procedure, mobile phase composition and conditions of the analysis were optimized.

3.1 Optimization of the sample pre-treatment

Metanephrines are extracted (using SPE) from untreated blood plasma samples. A range of commercially available SPE columns and several types of sorbents were tested. The sorbents were used for filling empty columns (volume 3 mL). The optimal sorbents to extract metanephrines from plasma samples were found. The SPE

procedure was also successfully optimized to achieve the highest possible yields of MN, NMN and 3-MT.

3.2 Optimization of UHPLC/Coulochem analysis

As the mobile phase were tested various types of buffers. The most suitable water for the preparation of the mobile phase for UHPLC/Coulochem was also chosen based on the tests. Now we use a mixed buffer adjusted to pH 2.94 (containing acetonitrile and water prepared in the Select Neptune Ultimate water purification system).

Metanephrines were separated on several different columns. The most suitable core-shell column was chosen according to tests. This column is heated to 28°C and a flow rate of a mobile phase is set at 0.5 mL/min.

A current-voltage curve of MN, NMN, 3-MT and HMBA was measured to optimize the potentials of the detector cells. The potentials are set at +400 mV (the conditioning cell), +100 mV (1^{st} electrode) and -350 mV (2^{nd} electrode).

3.3 Analysis of blood plasma samples

We analyzed hundreds of blood plasma samples (from laboratories of the First Faculty of Medicine (Charles University in Prague) and General Teaching Hospital in Prague) with determined values of metanephrines. Our results vary about 2-12 % from the Prague laboratories' results.

4 Conclusions

The developed method can substantially help identify persons who have the tumour pheochromocytoma. It is possible to distinguish blood plasma samples of healthy people from samples of people with PHEO. We are currently validating our method. The project ends in December 2014. Our kit for the determination of plasma metanephrines will be available in the beginning of 2015.

Acknowledgement

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P80 ZIC®-HYDROPHILIC INTERACTION CHROMATOGRAPHY SEPARATION OF PURINE AND PURINE BASES

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1 Introduction

Separation and determination of purine and purine bases is an interesting and challenging task because these compounds are involved in a variety of biochemical processes. It is not surprising that much labour has been spent on the study of their composition over the last decades [1]. These facts lead to the need for an inexpensive, quick and reliable method for the qualitative and quantitative analysis of purine and purine bases. A number of methods have been developed for purine and its derivatives, including voltammetry [2], spectrophotometry [3] and high performance liquid chromatography (HPLC) [4]. Among these HPLC is currently being used because it is reproducible, selective, sensitive and easily automated, particularly it can assay a series of purine derivatives simultaneously. While most commonly used methods apply reversed phase methods on classical C18 columns, we present in this application a method that makes use of the ZIC®-HILIC phase [5]. The great advantage of HILIC methods compared to RP methods is the ability to separate also very polar compounds.

2 Experimental

2.1 Instrumentation

HPLC system from Agilent Technologies was used for analysis. This system consisting of the following modules: vacuum degasser mobile phases (G1379B), dual high pressure binary pump (G1312B), autosampler (G1329B), a column thermostat (G1316B), DAD detector (G1315) and evaluation program Agilent ChemStation. The column employed for purines separation was a ZIC®-HILIC, 150 × 4.6 mm and 5 µm of particle diameter.

2.2 Chemicals

All chemicals were of the highest available purity. Stock standards of each analyte were purchased from Sigma (Steinheim, Germany). The analytes were prepared by separate in different solvents according to their solubility. All aqueous solutions were prepared at the desired concentrations in ultra-high-quality water Labconco (Water Pro PS, Cansas City, USA) and Milli-Q water produced by tandem of Labconco (Water Pro PS, Cansas City, USA) and Milli-Q (Millipore, Billerica, USA) water purification systems.

3 Results and Discussion

3.1 Optimization of the chromatographic separation

Four mobile phase gradients were evaluated in order to obtain the best separation of the nine analytes (caffeine, theobromine, purine, adenine, hypoxanthine, xanthine, guanine, isoguanine, uric acid). For the selection of the gradient, some chromatographic parameters as retention factor *(k)*, selectivity *(α)* and resolution of peaks *(R_s)* were calculated. In Fig. 1. is shown dependence of the retention factor *(k)* on the gradient program (*GR*). When gradient 1 and 2 were applied some analytes eluted at the same time and they overlapped so these gradients were discarded of the study. Moreover, gradient 2 caused analyte broadening indicating a lower efficiency of the column.

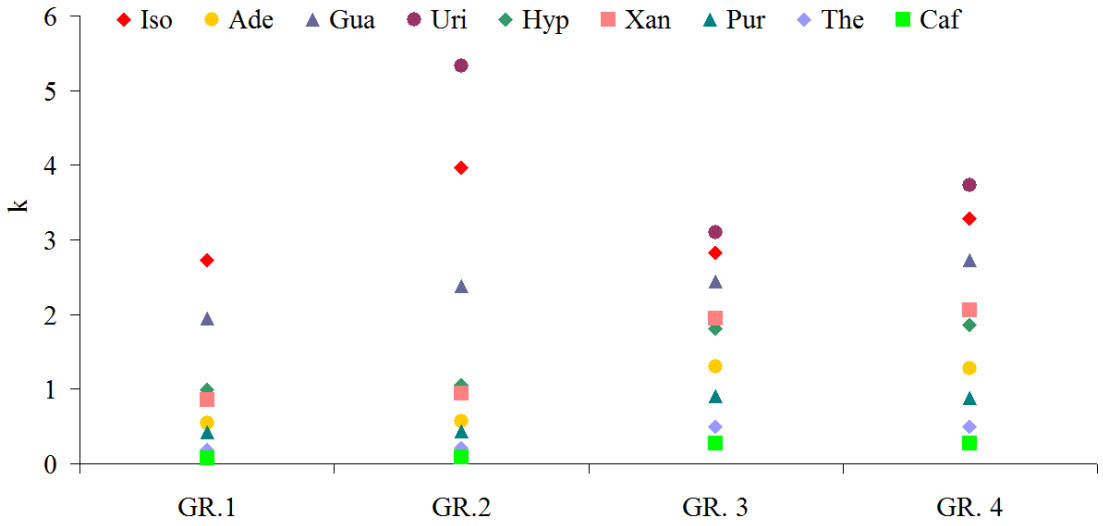

Fig. 1. Values of retention factor of the nine analytes at different gradients evaluated.

Selectivity and resolution parameters provide information about the separation of two compounds. While selectivity only takes *k* value to indicate separation of the two compounds, resolution is defined as the difference in retention times between the two peaks, divided by the combined widths of the elution peaks. Regarding to resolution values, gradient 4 (GR. 4) provided slightly higher values. Theobromine–caffeine pair was the only one that would not be able to quantification because overlapping was higher than 90 % (R_s < 1). The other resolution values were 1.5 or higher. The most suitable gradient for purine and purine bases separation was number

4 because of the good results achieved in terms of retention time, selectivity and resolution (Fig. 2.), without significant low efficiency respect to shorter gradients.

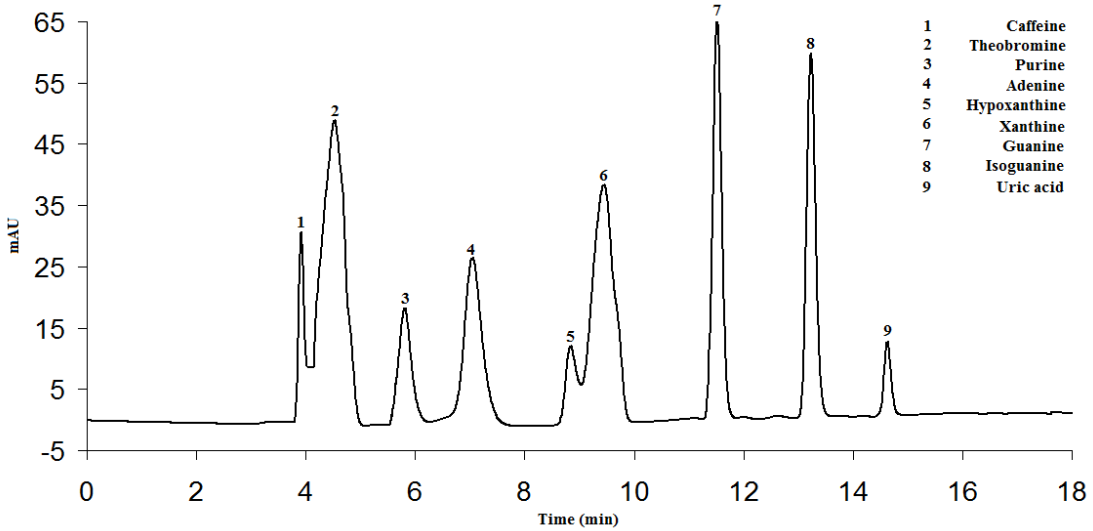

Fig. 2. HILIC separation of purines bases on ZIC®-HILIC column. Mobile phase: ammonium acetate, pH = 6.8 (A) and ACN (B); linear gradient of 90 % B at 0 min. to 55 % B at 15 min., returning to 90 % B at 20 min.; flow rate 0.5 mL min^{-1}; Column temperature 30 ± 0.1 °C; Injection volume: 10 µL; DAD detection wavelength 275 nm.

3.2 Limits of detection and quantification

The limits of detection (LOD) and the limits of quantification (LOQ) were calculated for each analyte using a signal-to-noise ratio S/N = 3 and S/N = 10, respectively (Table 1).

3.3 Precision

The repeatability (intraday), expressed as a relative standard deviation (RSD), was studied to obtain the method precision. Intraday analysis was established by the injecting of the standard solution mixture four times (n = 4) at the same day (Table 1).

Table 1. LOD and LOQ for the nine analytes.

	LOD (mg L^{-1})	LOQ (mg L^{-1})	RSD[a] (%)
Caffeine	0.40	1.40	0.90
Theobromine	0.50	1.60	0.50
Purine	0.70	2.30	0.70
Adenine	0.70	2.30	0.20
Hypoxanthine	1.20	4.10	0.10
Xanthine	0.20	0.80	0.00
Guanine	0.20	0.50	1.00
Isoguanine	0.30	0.90	0.40
Uric acid	3.10	10.40	4.80

[a] For peak area (n = 4)

4 Conclusions

This method can be successfully used in the separation of purine and purine bases by ZIC®-hydrophilic interaction chromatography. The LOD values were in the range 0.20–3.10 mg L^{-1} and LOQ range from 0.50 to 10.40 mg L^{-1}. All the RSD values were in the range 0.00–4.80 %, demonstrating that the method was precise. A required separation of analytes was achieved within 18 minutes.

Acknowledgement

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P81 IN VIVO ANALYSIS OF DICLOFENAC AND ITS HYDROXY-METABOLITES BY HPLC-MS

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1 Introduction

Drugs are principally eliminated from the body by enzymatic biotransformation reactions [1]. These reactions are mediated by various oxidation and conjugation enzymes in order to formation more hydrophilic metabolites. Metabolism is generally considered as a detoxification process [2]. Metabolism can be responsible for the problems with bioavailability, inter-individual variation, drug-drug interactions and idiosyncrasies. Therefore, the information related to the metabolic properties of drug is very important [1]. Met ID studies provide information on the sites or functional groups that need to be blocked or modified in order to improve metabolic properties of the drug. There is also possibility, that metabolite will be more pharmacologically active as parent compound, therefore metabolite identification is very important [3].

Diclofenac sodium is a non-steroidal anti-inflamatory drug of the phenylacetic acid class [4]. It is one of the most frequently administered drug relief in humans as well as veterinary medicine [5]. Diclofenac is extensively metabolized in humans to a number of hydroxylated metabolites which are found in plasma and urine in free and conjugated forms. The major diclofenac metabolite in plasma and urine is 4´-hydroxy(OH)-diclofenac and minor metabolites are 3´-OH-diclofenac and 5-OH-diclofenac [4]. For diclofenac analysis in different pharmaceutical preparations [6], biological [7, 8] and environmental [9] samples were developed numerous different methods such as liquid chromatography with UV or mass spectrometric detection [6, 9], gas chromatography-mass spectrometry [10], capillary electrophoresis [11] and some electrochemical methods [8, 12].

The aim of this work was analysis diclofenac and its hydroxy-metabolites in human urine samples by HPLC-MS.

2 Experimental

All HPLC-MS analyses were performed by using Shimadzu LCMS-IT-TOF™ (Shimadzu, Kyoto, Japan) equipped with electrospray ionization. HPLC experiments were performed on Ascentis C18 column (100x2.1 mm; 5 μm) (Sigma-Aldrich, Steinheim, Germany), using a gradient elution (water – acetonitrile) with a 0.2 ml/min flow rate. Gradient program was as follows: 0-1 min. - 10% ACN, 1-6 min. - 10-90%, 6.01-12 min. - 10% ACN. The column was thermostated to 40 °C. The $MS–MS^3$ experiments automatically acquired data within range of 50-1000 m/z in the positive and negative modes. Data acquisition and evaluation was performed using LCMS

Solution ver.3.4.151 (Shimadzu). For identification the potential metabolites of ibuprofen in human urine samples MetID program (Shimadzu) was used.

Formic acid, acetonitrile (LC-MS quality) and water (LC-MS quality) were purchased from Merck (Merck, Darmstadt, Germany). Standard of diclofenac was obtained from Cayman Chemical Company (Cayman Chemical Company, Tallinn, Estonia).

Diclofenac standard stock solution (1 mg/ml) was prepared by dissolving of 1 mg of standard in 1 ml of water (LC/MS quality). Working solution (c = 0.1 mg/l) was prepared by dilution of diclofenac standard stock solution with water (LC/MS quality). Samples of the remedy Flector® EP Rapid 50 mg (IBSA Institute Biochimique SA, Lugano, Switzerland) were obtained from the local pharmacy.

Urine samples were collected from five healthy volunteers in range 0 to 6 hours after oral use of 1 dose of Flector® EP Rapid (declared content of diclofenac sodium salt was 50 mg). All urine samples were diluted immediately after the collection with acetonitrile in a 1:1 (v/v) ratio, centrifuged during 5 minutes at 5000 rpm and filtered through a 0.45 µm filter (Millipore, Molheim, France). Subsequently, 10 µl of each sample was injected directly into the LC-ESI-IT-TOF MS analyzer.

3 Results and Discussion

Because of the nature of diclofenac, only MS spectra in negative ionization mode are shown in this work. Urine samples were obtained form five healthy volunteers, two women and thee man in range 23-55 ages. From each volunteer was obtained blank urine sample and consequently each volunteer take one dose of Flector® EP Rapid. Collecting of urine samples was stopped after 6 hours because of diclofenac half-life (1-2 hours [13]). On Fig. 1 are shown TIC and XIC records obtained form HPLC-MS analysis of urine sample of volunteer no. 1 after 3 hours after administration of one dose of Flector® EP Rapid. For metabolite identification was used program MetID Solution. On Fig. 2 is showed XIC record of potential metabolite of diclofenac marked with MetID Solution as a hydroxy-diclofenac and MS spectrum with molecular ion of hydroxy-diclofenac (m/z 310.0052) obtained from HPLC-MS analysis of urine sample obtained from volunteer 4 after 4 hours after administration of one dose of Flector® EP Rapid.

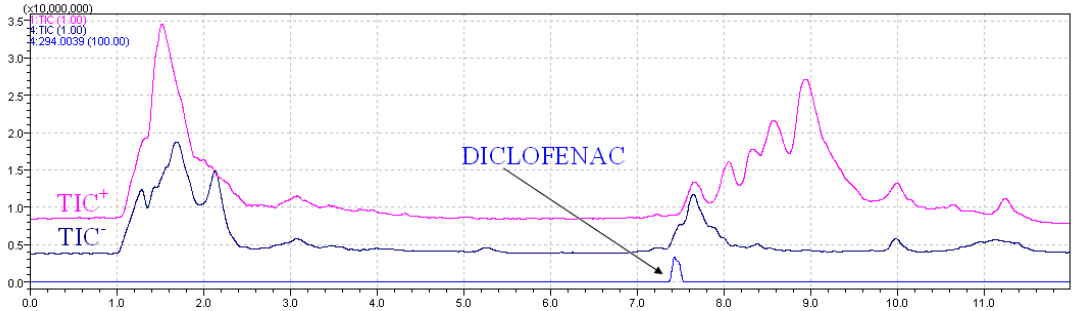

Fig. 1. TIC and XIC records obtained form HPLC-MS analysis of human urine.

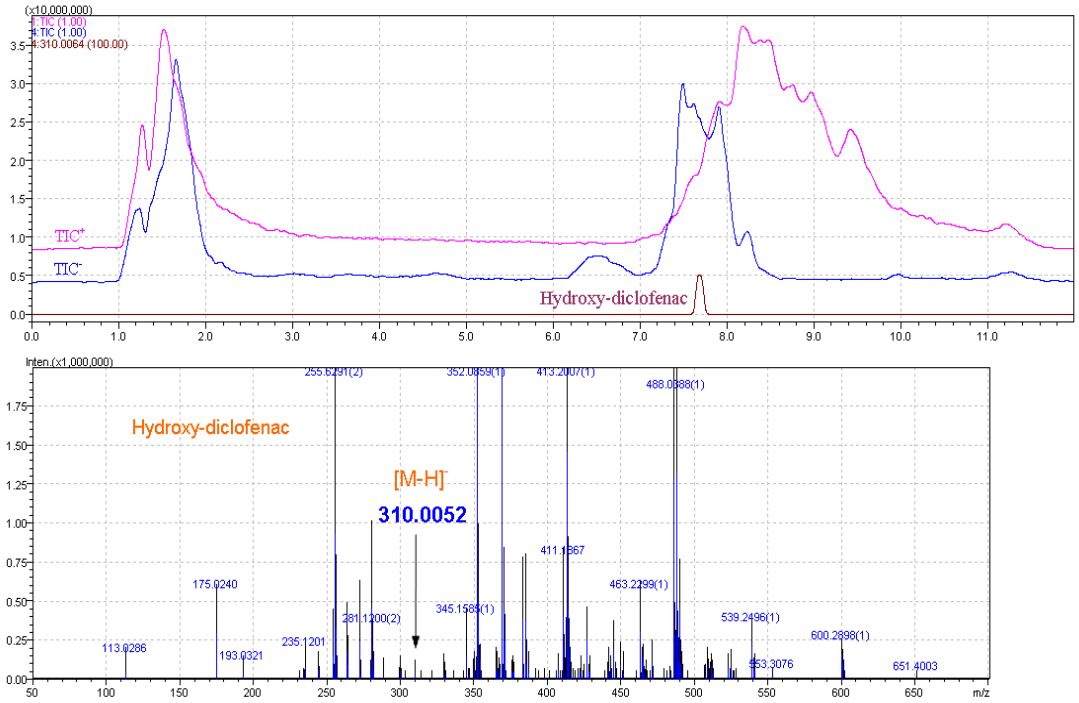

Fig. 2. XIC record and MS spectrum of potential metabolite of diclofenac.

4 Conclusions

HPLC-MS is very suitable method for analysis of drugs and their metabolites. In our work we analyzed and identified diclofenac and its hydroxy- metabolites in urine samples of five healthy volunteers after administration of one dose Flector® EP Rapid.

Acknowledgement

This contribution is the result of the project implementation (ITMS 26240220061) supported by the OPRaD funded by the ERDF. This work was financially supported by the grant of the Slovak Research and Development Agency (APVV-0583-11).

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P82 CAPILLARY ELECTROPHORESIS WITH NATIVE FLUORESCENCENCE DETECTION IN THE WIDE BORE CAPILLARY

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Summary
Combination of capillary electrophoresis with fluorescence detection is a powerful analytical tool for analysis of biological samples. This work dealt with the optimization of fluorescence detector settings to achieve the lowest detection limits for the analytes in the separation system with plastic capillary tube of 0.3 mm I.D. Photomultiplier and xenon lamp was connected to the detection cell in right angle configuration by fiber optics. Tryptophan, N-acetyl tryptophan and fluorescein were selected as model analytes with intrinsic fluorescence. Electrophoretic separations were performed in a low conductivity electrolyte at pH 9.2 and suppressed electroosmotic flow.

1 Introduction

Capillary electrophoresis (CE) combined with UV absorbance detection has a poor concentration sensitivity and, in addition, the deteriorating effect of the sample matrix on the separation efficiency typically can occur in multicomponent samples. These difficulties can be overcome by the use of a more sensitive detection technique, a sample clean-up or on-capillary pre-concentration. Fluorescence detection (FLD) in a comparison with UV absorbance detection is more sensitive and, at the same time, more selective. CE-FLD can easily be used for analysis of compounds with native fluorescence. Detection of analytes without fluorophore in CE-FLD requires sample

derivatization [1]. An appropriate excitation source is crucial to obtain sensitive FLD. Excitation sources that allow most flexible wavelength selection are high-pressure xenon, mercury–xenon, or deuterium lamps. Lasers produce an intense beam of light of a very pure single color. Light-emitting diode (LED) provides a broader spectral bandwidth than lasers, it nevertheless becomes an attractive alternative to lasers as an excitation source. The optical configuration of a fluorescence detection cell requires an efficient capture emission light of excited analytes, while excitation light and scatter is rejected [2].

Analytical benefits associated with the use of capillary tubes of larger I.D.s in CE were shown by Kaniansky et al. [3].

The present work was aimed at investigating some factors which contribute to sensitivity of FLD in CE performed in plastic capillary tubes of larger I.D. In this study, a model analytes with native fluorescence (tryptophan, N-acetyl tryptophan and fluorescein) were used.

2 Experimental

2.1 Instrumentation

An electrophoretic analyzer EA-101 (Villa-Labeco, Slovakia) working in a hydrodynamically closed single-column arrangement was used for the CE separations. The capillary column made of fluorinated ethylenepropylene copolymer (FEP) with 0.3 mm I.D., 0.7 mm O.D. and 200 mm length was equipped with detection cell enabling the connection of fiber optic probes in different angles. In this work the right angle configuration was used. Separations were monitored by photomultiplier (PMT III, J&M, Germany) connected to the detection cell placed 160 mm from the injector with fiber optic probe via long wave pass filter (305FG01, Andover Corporation, USA). Xenon lamp (FL3095SL, J&M) was used for excitation at 290 nm. The sample injection was performed by a CZE injection valve (Villa-Labeco) with 220 nL fixed volume and 3 mm plug length.

2.2 Buffer and standards preparation

CE separations were performed in alkaline background electrolyte system at pH 9.2 consisting of 30 mmol/l glycine, 15 mmol/l bis-tris propane and 0.1% (v/v) hydroxyethylcellulose served as a suppressor of electroosmotic flow.

The stock solutions of tryptophan, N-acetyl tryptophan and fluorescein were prepared at a 100 mg/L concentration and then diluted with ultra-pure water.

3 Results and Discussion

Typically, tryptophan has a wavelength of maximum absorption of 280 nm and a fluorescence emission peak ranging from 300 to 350 nm depend on the polarity of solution. The wavelength of excitation light beam was set to a minimum enabled by the used instrumentation (290 nm). A long wave pass filter (305 nm) was used in the pathway of emission light to eliminate the excitation light.

A low conductivity background electrolyte with alkaline pH was selected because of tryptophan ionization and voltage-current characteristics. In the

optimization process of detection parameters the binary mixture of tryptophan and N-acetyl tryptophan was injected in the CE capillary.

Impact of different settings of detection system, including time of data collection, voltage on the photomultiplier tube and gain, on the limit of detection (LOD) was tested. Data collection time was tested in the range of 50 to 1000 ms. The time 200 ms was chosen on the basis of the shape of peaks and signal-to-noise ratio (even though that the detection at other times was also acceptable).

Voltage on the photomultiplier tube was tested in range of 600 to 900 V. Stable signal was obtain at 800 and 900 V. Unexpected decrease or increase of baseline between run-to-run was observed at 600 and 700 V. The usage of appropriate gain is very important, because the intensity of the measured radiation must be kept under the maximum intensity. In this work, gain was set in the range 20 to 80 in a dependency of the used voltage on photomultiplier tube.

The LOD values were calculated according to Foley – Dorsey relation [4]. In CE separations under optimized FLD detection conditions the calculated LOD was 4.8 and 4.1 μmol/l for tryptophan and N-acetyl tryptophan, respectively.

Fluorescein with considerably higher emission of fluorescence serves for a comparison of detection possibilities. Under identical detection conditions than in CE separation of amino acids the calculated LOD for fluorescein was 133 nmol/l.

4 Conclusions

The results achieved in CE separations with the used detector instrumentation and detection cell indicate, that this set-up is a promising analytical tool from the point of sensitivity. Selectivity of FLD can be useful in analysis of biological samples.

Acknowledgement

This work was supported by the Slovak Research and Development Agency (APVV-0259-12 and APVV-0583-11) and the Grant of Comenius University in Bratislava (UK/496/2014).

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P83 KINETIC STUDY OF KETAMIE N-DEMETHYLATION MEDIATED BY ON-LINE ENANTIOSELECTIVE CAPILLARY ELECTROPHORETIC METHOD

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Summary
Enantioselective discrimination is an important phenomenon influencing pharmacokinetics and pharmacodynamics of chiral drugs in humans and animals. Metabolism appraisal of single enantiomers of every drug candidate compound thus constitutes the inseparable part of LADME/tox screening. Capillary electrophoresis represents a promising technique in the field of *in vitro* enzyme assays due to miniscule sample consumption, simple implementation of chiral separations and high-throughput by automation. An optimized and economical capillary electrophoretic method for on-line studies of the enantioselective drug metabolism mediated by cytochrome P450 3A4 enzyme (CYP3A4) is introduced.

1 Introduction

Pharmacokinetic and pharmacodynamic properties of a chiral drug can significantly differ between application of the racemate and single enantiomers. Since approximately 56 % of currently marketed drugs are chiral compounds [1], screening of the single enantiomers of a new drug candidate for the LADME/tox characteristics became a routine part of drug discovery and development. Biotransformation significantly influences drug actions in an organism; metabolism studies therefore constitute a crucial part of these tests. Capillary electrophoresis (CE) represents a promising technique to assess the fate of drug enantiomers *in vitro* and *in vivo*. Chiral separations are simply implemented with commercial instruments featuring fused-silica capillaries, provide a high separation efficiency, require minuscule amounts of sample and reagents, offer high throughput via automation and permit analyte monitoring with a variety of detection methods. Furthermore, on-line CE methods can use a fused-silica capillary as a reaction vessel thus integrate the incubation of an enzymatic reaction, separation of reaction products and their detection and quantitation into a single fully automated run. The goal of this study was to continue in previous efforts [2] and develop an on-line CE method enabling conduction of kinetic and inhibition studies of the enantioselective drug metabolism mediated by CYP3A4. N-demethylation of ketamine was selected as a model system.

2 Experimental

A ProteomeLab PA 800 CE System (Beckman Coulter, Fullerton, CA, USA) was used to perform all analyses. Both enzymatic reaction and separation of reaction products were carried out at 37 °C in a 50 µm id, 375 µm od fused-silica capillary (54 cm effective length). The in-capillary reaction occurred via alternate hydrodynamic introduction of 4 short plugs comprising 2 mM NADPH and substrate, and 3 short plugs of 400 nM CYP3A4. These solution were prepared in 100 mM potassium phosphate (pH 7.4) used as an incubation buffer. Each plug of ketamine and NADPH solution was injected for 3 s into the capillary at a pressure of 0.5 psi and each plug of CYP3A4 solution was introduced by application of a negative pressure of -0.5 psi for 4 s. The reaction mixture was incubated for 8 min. The enzyme reaction was terminated by concomitant application of -20 kV (-312.5 V/cm, negative polarity) and a positive pressure of 0.2 psi (small buffer flow towards the anode) and separation of the reaction mixture components. The background electrolyte was composed of 50 mM TRIS-phosphate buffer (pH 2.5) and 3 % w/v of highly sulfated γ-cyclodextrin as chiral selector. The analytes were detected using the on-column PDA UV-VIS detector set to 195 nm.

3 Results and Discussion

Since extensive numbers of drug candidate compounds are tested within early stages of a new drug development, a generic diffusion-based procedure which enables mixing of virtually any compound and CYP into reaction mixture was adopted [3]. The final method was then optimized so that it enables the baseline separation of the enantiomers of both substrate and reaction product (Fig. 1).

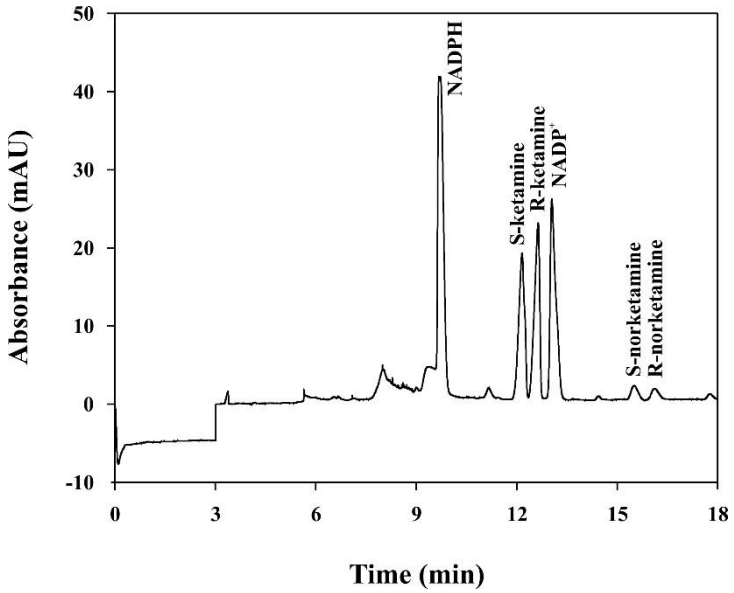

Fig. 1. Typical electropherogram obtained after the in-capillary reaction with 200 nM CYP3A4, 400 µM racemic ketamine and 1 mM NADPH. For incubation and separation conditions see section 2.

After the optimization was finished, the method was thoroughly validated. Since all obtained specifications were considered to be excellent, the final method was used for kinetic study of CYP3A4 reaction with ketamine as substrate. The determination of the apparent Michaelis-Menten constant, apparent maximum reaction velocity and Hill coefficient was obtained with various amounts of racemic ketamine and its single enantiomers in the reaction mixture. Data obtained were curve-fitted using nonlinear regression analysis according to the Hill equation (Fig. 2).

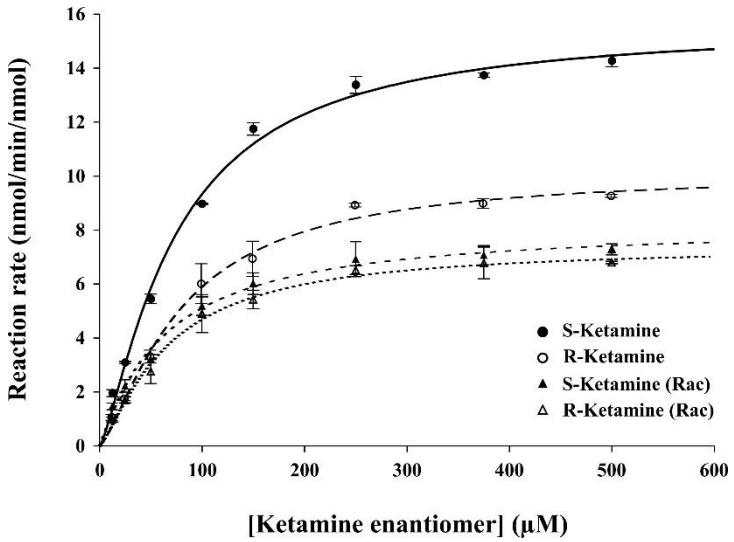

Fig. 2. Norketamine formation via CYP3A4 for racemic ketamine and its single enantiomers. Line graphs are predicted based on nonlinear regression analysis using the Hill equation. Rac refers to incubations with racemic ketamine.

Determined values of the basic kinetic parameters were in a good agreement with literature data obtained using different techniques [2,4,5]. The same is valid for the observation that the formation rate of S-norketamine was significantly higher compared to that of R-norketamine. Results of paired Student's *t*-test proved that the N-demethylation of ketamine mediated by CYP3A4 proceeds stereoselectively in the incubations of both racemic and single enantiomers substrates.

4 Conclusion

An improved CE method for on-line investigation of the enantioselective CYP3A4 mediated drug metabolism based on transverse diffusion of laminar flow profiles was developed. Since diffusion is the inherent property of all molecules, the adopted procedure enables addition of virtually any compound into the reaction mixture without need of additional optimization of the mixing conditions. Obtained values of basic kinetic parameters are comparable with literature data and thus prove the practical applicability of the method. Minimal consumption of enzyme and other chemicals, fully automated analyses and simple transfer to a multi-capillary format

make this approach a promising tool for high-throughput screenings of potential drug candidates in the early stages of new drug developments.

Acknowledgement
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P84 USE OF ARTIFICIAL NEURAL NETWORKS FOR OPTIMIZATION OF BIOGENIC AMINES DERIVATIZATION

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Summary
Biogenic amines as biologically active molecules with potential toxicological dangers are often subject of interest in food analysis. Since these substances do not exhibit significant fluorescence nor absorbance, derivatization is necessary for analysis in combination with RP-HPLC-UV setup. The use of experimental design (ED) in combination with artificial neural networks (ANN) for optimization of dansyl derivatization conditions for seven biogenic amines is presented. Four key factors were studied – concentration of derivatization reagent, buffer pH, temperature and incubation time. With the use of ED-ANN approach, optimal conditions for dansyl chloride derivatization were found and experimentally confirmed.

1 Introduction

Biogenic amines are low-molecular organic bases. These nitrogenous compounds are formed mainly by microbial decarboxylation of the free amino acids in foodstuff [1]. The determination of biogenic amines is of great interest because it is possible to use them as indicators of food quality, freshness or spoilage. Since high amounts of these

organic compounds are connected with toxicological dangers, importance of monitoring their levels is evident [2, 3].

Most biogenic amines are molecules with neither fluorescence nor strong enough absorbance in the UV-Vis region. Therefore derivatization is necessary for the detection. One of the most widely used reagents for analysis of biogenic amines by means of RP-HPLC is dansyl chloride (DCl) [4].

In order to increase the sensitivity, it is necessary to optimize the conditions of the dansylation. Several factors influencing yields of the derivatization reaction have to be considered – concentration of DCl, pH, temperature and incubation time [5, 6]. Optimization using single variable approach might be time consuming and it is unlikely to discover the optima in the case that the factors are correlated [7]. In this work ED-ANN approach is used to find experimental conditions that will allow fast and reliable amine derivatization (optimal conditions). The sum of peak areas for all amines in the reaction mixture obtained by subsequent HPLC analysis was considered for the optimization [8, 9].

2 Experimental

2.1 Chemicals

Seven biogenic amines – cadaverine (CAD), 1,7-diaminoheptane (DAH), histamine (HI), 2-phenylethylamine (PH), putrescine (PUT), tryptamine (TR) and tyramine (TY); HPLC grade acetonitrile (ACN) and dansyl chloride (DCl) were purchased from Sigma-Aldrich (USA). Aqueous ammonia, hydrochloric acid and sodium hydroxide were obtained from Lach-Ner (Czech Rep.). Sodium bicarbonate was supplied by Lachema (Czech Rep.).

2.2 Derivatization reaction

Standard solution was prepared by dilution of biogenic amines (1 mg/mL each) in 10mM HCl freshly before each use. For the derivatization reaction 100 μL of the diluted standard solution (10 μg/mL each), 150 μL of 0,5M sodium bicarbonate buffer (the tested pH range was 9–13.5) and 100 μL of DCl in ACN (concentrations 2.5–20 mg/mL) were mixed. The mixtures were then incubated in thermostat (TK-1c, KEVA, Czech Rep.) for desired time. After the incubation period, the excess of dansyl chloride was removed by addition of 50 μL of concentrated ammonia. After 5 min, 10 μL of the dansylated solution was injected onto the chromatographic column (Watrex 250 × 4 mm, Reprosil 100 C18 with 5μm particles). The separation was carried out using RP-HPLC system Watrex (Czech Rep.) in isocratic elution mode (mobile phase ACN/water – 70/30) with the flow rate 0.5 mL/min. Absorbance was measured at 254 nm.

2.3 Experimental design

Reduced central composite design for 4 factors (concentration of DCl, pH, temperature and incubation time) was selected to cover the factor space and provided initial screening. 18 chromatograms were obtained. Factors were used as inputs and normalized peak areas as outputs for ANN training. To avoid overtraining, 3 cases

randomly selected from the used ED (experimental conditions and corresponding peak areas) were used for verification. With the rough knowledge of the factor space it was possible to use another central composite design (17 training cases) that was more focused towards the expected optimum. Additional 3 cases, obtained by measurement at conditions randomly selected from the design area, were used as verification.

3 Results and Discussion

ANN evaluation was carried out using Trajan 3.0 (Trajan Software Ltd., UK). First, optimal structure of the ANN for the initial experimental design was searched. Network with 3 layers was found to be the most suitable. The structure consisted of 4 input neurons, 5 neurons in the hidden layer and 7 output neurons (4-5-7). According to the ANN prediction it was apparent that the best results were yield at temperature of 60 °C. However, the predicted reaction times were overly high for routine analysis (85 min).

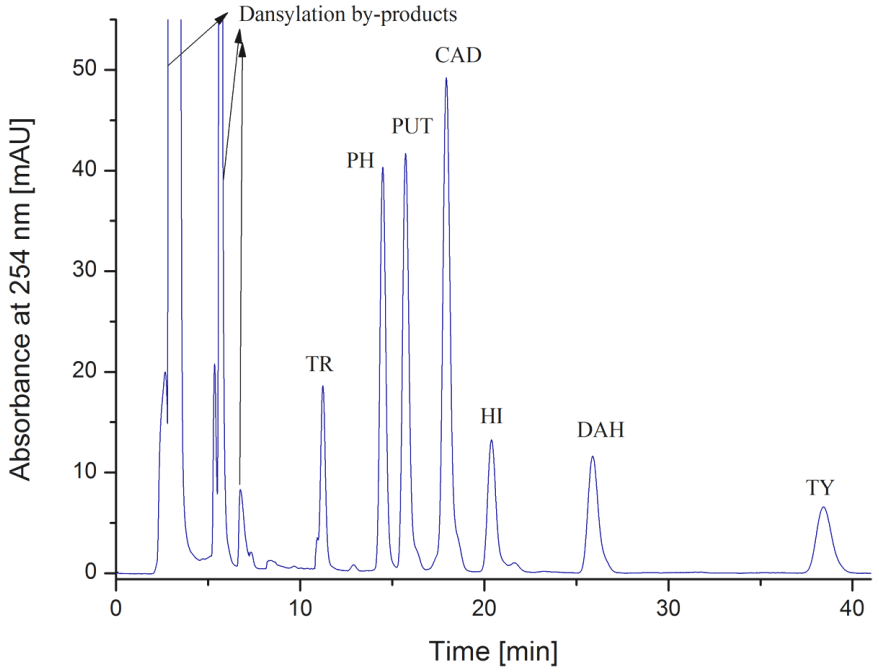

Fig. 1. Chromatogram of dansylated biogenic amines – tryptamine (TR), 2-phenylethylamine (PH), putrescine (PUT), cadaverine (CAD), histamine (HI), 1,7-diaminoheptane (DAH) and tyramine (TY), analyzed using RP-HPLC-UV.

In order to reduce the reaction time, new experimental design was constructed in the previously predicted experimental region. This new design was simplified by setting the temperature constant (60 °C) and new ANN was constructed. In this case, the optimal ANN architecture was (3-7-7). The network was used to predict the optimal dansyl derivatization conditions: reaction time 40 min, buffer pH 12.5, DCl concentration 11 mg/mL and temperature 60 °C. The separation of sample prepared using the above mentioned conditions was performed using RP-HPLC (see Fig. 1) and the suitability of the method for biogenic amines derivatization was confirmed.

Sample volume needed for the dansylation was 100 μL and HPLC analysis of 10 μL of dansylated mixture took 40 min. The time required for separation can be further improved by optimization of the HPLC separation by ED-ANN approach.

4 Conclusions

Biogenic amines are very often subject of interest in the field of food analysis. For analyses using RP-HPLC with UV-Vis detection, derivatization is necessary. In this work, ED-ANN approach was used to find the optimal conditions of amine derivatization using dansyl chloride. Seven biogenic amines were analyzed – cadaverine, 1,7-diaminoheptane, histamine, 2-phenylethylamine, putrescine, tryptamine and tyramine. Four key factors were studied – concentration of derivatization reagent, buffer pH, temperature and incubation time. With the use of ANN, optimal conditions were found and experimentally confirmed. The optimized derivatization method seems to be suitable for analysis of biogenic amines and has a potential to be used in real sample analysis.

Acknowledgement

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P85 DETERMINATION OF 3-NITROTYROSINE IN URINE BY MICROCHIP ELECTROPHORESIS

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Summary
Simple, sensitive and fast microchip electrophoretic (MCE) method was developed for determination of 3-nitrotyrosine (3-NT) in human urine samples. Biological samples were analyzed by MCE with coupled conductivity and spectrophotometric detection at wavelength of 400 nm. Limit of detection (LOD) and limit of quantification (LOQ) were determined as 4.2 nM and 13.9 nM, respectively. The average concentration of 3-NT in human urine sample was 1.15 μM with a relative standard deviation of 8.21%.

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. One of the most significant markers of oxidative damage of proteins is 3-NT and it generally exists in plasma and urine. Therefore it is important to monitor the level of 3-NT in tissues and biological fluids to evaluate oxidative stress. A variety of analytical methods have been developed for detection and quantification of 3-NT, e.g. ELISA assays [1], GC [2], LC-ECD [3], LC-UV [4], HPLC [5], CE[6], etc.

Analytical techniques with high separation efficiency and good detection sensitivity are needed as the concentration of 3-NT is expected to be very low in urine samples. MCE has many advantages, such as high separation efficiency, low sample consumption, low costs and a wide range of application. Due to mentioned properties MCE can be used for trace analysis of biological samples.

2 Experimental

Chemicals used for the preparation of electrolyte and model sample solutions were obtained from Sigma-Aldrich (Seelze, Germany).

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. Stock solution of 3-NT was prepared at 10 mM concentration. Urine samples collected in the morning from 1 volunteer were diluted with deionized water and stored at -30°C. No other sample pretreatment was used before the analysis.

Poly(methylmethacrylate) (PMMA) microchip with coupled separation channels (CC) and on-column conductivity detectors (Merck) used in this work was made by technological procedure described in detail in the literature [7]. For determination of 3-NT was used WellCrom Filterphotometer K-2001 with wavelength of 400 nm (Knauer, Berlin, Germany).

3 Results and Discussion

The CZE separations were performed with suppression of electroosmotic and hydrodynamic flow. The PMMA microchip with conductivity detection in the first separation channel and spectrophotometric detection at the end of the second separation channel was used. For data evaluation the second detection cell on the microchip was used. The wavelength of spectrophotometric detection was set on a 400 nm due to absorbance maximum of 3-NT (426 nm) [8]. Using the background electrolyte with pH of 9 and maintained voltage at 4 kV, total time of analysis was less than 700 s. LOD of 3-NT was determined as 42.2 nM using Foley-Dorsey method [9]. This value is significantly lower than those referred to in Table 1, where the LOD values of other methods are summarized. LOQ of 3-NT was evaluated as 13.93 μM using an equation: LOQ = 3.3 × LOD.

Table 1. Comparison of published LOD for 3-NT.

LOD [μM]	LOD [mg/L]	Reference
1,77	0,4	6
0,07	$1{,}16 \times 10^{-2}$	8
0,002	$4{,}52 \times 10^{-4}$	10
3,097	0,7	11
0,004	$9{,}54 \times 10^{-4}$	Our method

Urine sample was directly analyzed by MCE with spectrophotometric detection at 400 nm wavelength. The concentration of 3-NT (1.15 μM) in the analyzed sample (Fig. 1a) was found by method of standard addition. Fig. 1b represents the addition of 2 μM 3-NT. RSD values of peak height and peak area of 3-NT were about 8%.

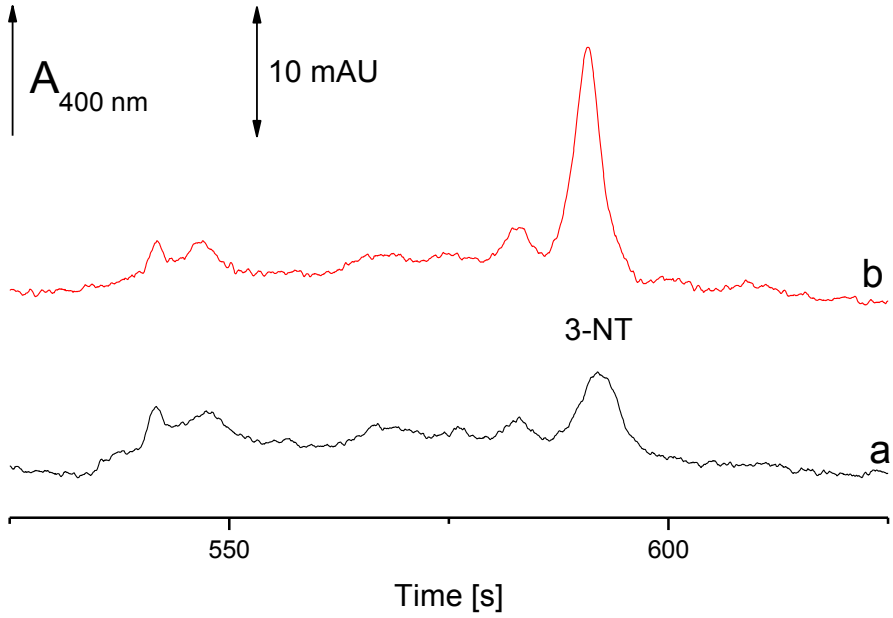

Fig. 1. Electrophoreograms of urine sample (a) and urine sample with addition of 2 μM 3-NT (b).

4 Conclusions

We have developed fast and sensitive method for trace determination of 3-NT in human urine. Our approach based on MCE with spectrophotometric detection brings promising results in term of LOD for 3-NT (less than 50 nM). Moreover, the total time of analysis in this work (700 s) is much less than those for other methods for 3-NT analysis, such as for LC-ECD (48 min), LC-UV (21 min) or ELISA analysis (4 hours). Short time analysis and good sensitivity make our method a highly efficient and easy applicable method for determination of 3-NT in biosamples.

Acknowledgement

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P86 ZIC-HILIC MONOLITHIC CAPILLARY COLUMN COUPLED WITH MALDI-MS: A TOOL FOR GLYCAN ANALYSIS

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Summary
In this contribution, we report analysis of glycans enzymatically released from bovine ribonuclease B (RNase B) and human immunoglobulin G (hIgG) combining glycan separation using the synthesized zwitterionic silica-based monolithic capillary column and off-line MALDI-MS detection.

1 Introduction

Glycoproteins play an important role in various biological functions and thus represent attractive substances for pharmaceutical industry. However, 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 HILIC with ESI/MS detection has been recognized as an effective tool for glycan analysis [1]. In this contribution, we report the analytical method combining HILIC separation using a zwitterionic silica-based monolithic capillary column and off-line MALDI-MS detection.

2 Experimental

Glycoproteins were deglycosylated using a soluble peptide-N-glycosidase F [2]. Sulfoalkylbetaine-modified monolithic silica-based capillary column (150×0.1 mm) was prepared according to the procedures described previously [3]. Separations were performed in a gradient mode using a simple chromatography platform [4-6] that was connected to the lab-made MALDI deposition device. The S-shaped mobile phase gradient was created by sucking of weak mobile phase (30 µl, 200 µl/min, of 80% v/v acetonitrile/10 mmol/l CH_3COONa, pH 6.5) into the 500×0.45 mm steel capillary filled with strong mobile phase (65% v/v acetonitrile/10 mmol/l CH_3COONa, pH 6.5). The glycan samples were diluted in a weak mobile phase and a sample volume of 0.1 µl was injected. Elution was performed at 1µl/min and 27 °C. Effluent was continually mixed with a solution of 20 mg/ml super-DHB dissolved in 5mmol/l $NaHCO_3$ in the 1:1 ratio and spotted on the MALDI plate. Total deposited volume per one spot was 1 µl. Mass spectra were acquired by AB SCIEX TOF/TOF 5800 system in a reflectron mode. The individual glycans were detected as sodium adducts in a positive mode.

3 Results and Discussion

Glycans containing the numerous hydroxyl groups are highly hydrophilic compounds which limits the use of traditional chromatographic stationary phases such as reversed-phase sorbents for their enrichment and fractionation. In contrast, HILIC involving solvents with high content of organic component (40-97% ACN) in water is an attractive chromatographic mode for the analysis of the glycoconjugates.

The capillary column used in this study was obtained by grafting a polymeric layer of [2-(methacryloyloxy)ethyl]-dimethyl-(3-sulfopropyl)-ammonium hydroxide to silica-based monolithic support which was prepared by acidic hydrolysis of tetramethoxysilane in the presence of polyethylene glycol and urea.

Figure 1 shows the MALDI/MS spectra of glycan mixture released from (a) RNase B and (b) hIgG). The glycan mixtures were separated on the synthesized sulfoalkylbetaine-modified monolithic silica-based capillary column in a gradient HILIC mode, continually mixed with a matrix solution and spotted on the MALDI plate. The mass spectra of separated glycans are shown in Fig. 1c (RNase B) and Fig. 1d (hIgG).

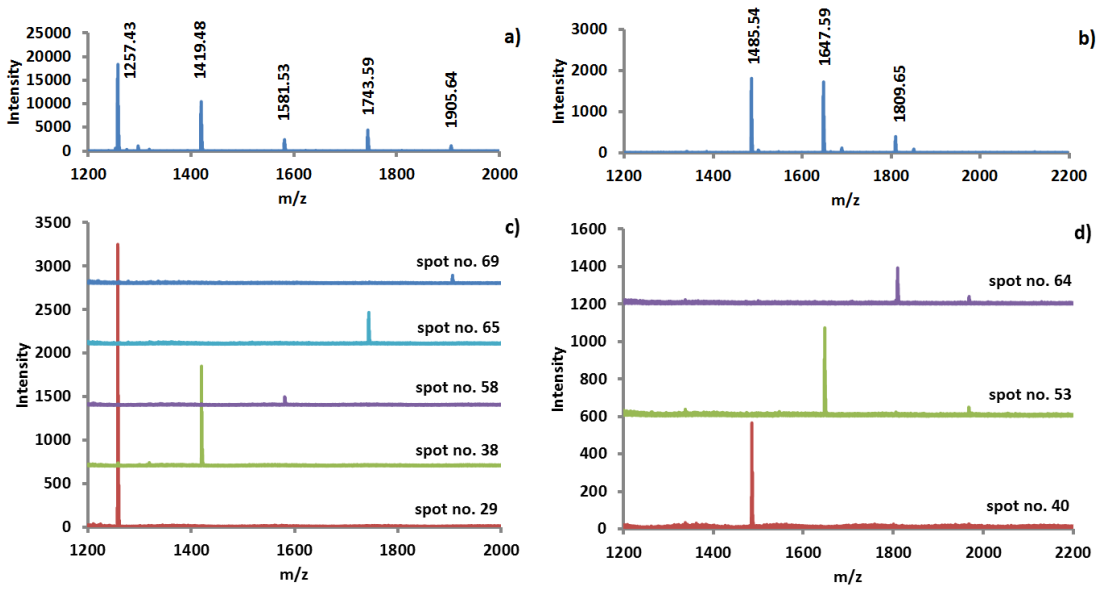

Fig. 1. MALDI/MS spectra of glycans released from (a) RNAse B and (b) hIgG. The individual fractions of (c) RNase B and (d) hIgG glycans obtained by HILIC separation.

4 Conclusions

Glycans released from RNAse B and hIgG, having insignificant retention on a reversed phase columns, were effectively retained and separated on a sulfoalkylbetaine-modified monolithic silica-based capillary column and detected in off-line mode by MALDI/MS.

Acknowledgement

This work was financially supported by the European Social Fund and the state budget of the Czech Republic (CZ.1.07/2.3.00/20.0182), the Grant Agency of the Czech Republic (14-06319S), the Grant of Ministry of Interior (VG20112015021), the Institute of Analytical Chemistry, v. v. i. (RVO68081715) and the Ministry of Education, Youth and Sports of the Czech Republic (FCH-S-14-2487).

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P87 FIRST INSIGHT INTO PHARMACOKINETICS OF DPC - A NOVEL IRON CHELATING ANTI-CANCER THIOSEMICARBAZONE

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1 Introduction

Cancer is considered by the majority of authorities around the globe one of the leading causes of death [1]. Despite the variety of antineoplastics developed over the past decades cancer still remains difficult to manage in clinical practice. Some of the reasons for that are resistance of cancer cells towards these drugs and fast metastatic dissemination, the latter being the main cause of high mortality rates. Therefore there is a constant need for novel, more effective and less toxic therapeutics to battle the illness.

Iron (Fe) is an essential micronutrient involved in cellular metabolism and proliferation. Thus targeting intracellular Fe ions, especially in cells with high Fe demands – such as cancer cells – is a promising approach for cancer treatment. Thiosemicarbazone Fe chelators have been proven in numerous *in vitro* and *in vivo* studies to be greatly efficient in suppressing growth of many cancerous cell lines as well as restricting their metastatic spread. It has been shown that thiosemicarbazones inhibit RNA-reductase, a key enzyme in cellular proliferation. In addition, the complexes with Fe and copper (Cu) undergo redox cycling, producing highly cytotoxic ROS (reactive oxygen species). Additionally, they also up-regulate expression of a strong metastasis-suppressor gene (NDRG-1).

The current lead compound among anti-cancer thiosemicarbazones is di(2-pyridyl)ketone-4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC). This compound is selectively toxic towards cancer cells as was documented in several cancerous and non-cancerous cell lines. With this favourable toxicity-efficacy profile, it is a promising compound with high hopes for shifting from preclinical to clinical trials. Nonetheless, pharmacokinetics (PK), one of the important characteristics of a novel drug candidate, need to be assessed prior to initiation of the clinical trials. The only available *in vivo* PK data for this class of thiosemicarbazones come from a study with a structurally related thiosemicarbazone 2-benzoylpyrine-4-ethyl-3-thiosemicarbazone (Bp4eT) [2].

The aim of this work was to develop and validate a UHPLC-MS/MS method for determination of DpC in plasma and utilize it in a PK experiment.

2 Experimental

This work was performed on Shimadzu Nexera system coupled with Shimadzu LCMS 8030 triple quadrupole mass detector. Electrospray in positive mode was employed as ionization technique. All analyses were carried out using Acquity C18 BEH analytical column with the same type guard column. The mobile phase consisted of aqueous ammonium formate (2 mM) solution with K_2EDTA (5 µM) (A) and acetonitrile (B) in a gradient mode. The plasma samples were treated with protein precipitation followed by liquid-liquid extraction. The organic extracts were then dried under a gentle flow of nitrogen and reconstituted in 250 µM K_2EDTA in 50% acetonitrile. The UHPLC-MS/MS method was validated over a range of 0.15-3 µM according to the FDA guidelines for Bioanalytical Methods Validation in respect to selectivity, linearity, precision, accuracy, stability, dilution integrity and matrix effects [3].

Male Wistar rats (n=6) were administered 2 mg/kg of DpC intravenously. Plasma samples were taken in predefined intervals over 6 hours and frozen until analysis. An additional *in vivo* experiment lasting 30 hours was performed (n=8) in order to describe the elimination phase properly. The PK parameters were calculated using the Kinetika 5.0 software.

3 Results and Discussion

At first, we developed an LC-MS/MS method for determination of DpC in plasma. It was found that analysis of DpC is complicated by strong chelation properties of this compound that precluded sensitivity and reproducibility assay using conventional mobile phases. Hence, the following procedure was developed to overcome this issue: 1) A new column was flushed with EDTA aqueous solution (2 mM) with acetonitrile (90:10) prior to first analysis. 2) Reconstitution solvent contains 250 µM EDTA. 3) Mobile phase contains trace amount (5 µM) of EDTA. 4) A solution of 3.5 mM EDTA in mobile phase is injected onto the column before every run. Generally, it is advisory to avoid use of non-volatile additives in LC-MS analysis, as these are documented to cause ion-suppression, clogging of the ion source or contamination of the MS instrument. However, it was recently shown that trace amount of EDTA in the mobile phase (5 µM) does not cause any detrimental effects on the instruments, while improving analysis of iron chelators significantly [4].

Protein precipitation (PP), liquid-liquid extraction (LLE) and solid-phase extraction (SPE) were tested to treat plasma samples. However, all these techniques failed either in extraction recovery or in reproducibility. A significant enhancement of both parameters was reached using a combination of PP with acetonitrile and LLE in dichloromethane where an acceptable reproducibility with recovery over 70% was obtained. All validation parameters reached the recommended values [3].

UHPLC-MS/MS method was eventually utilized in determination of concentration-time profile in plasma after administration of DpC to rats in a PK study. The obtained data were applied for evaluation of basic pharmacokinetics parameters. However, the significant difference (> 35%) between the area under the curve until

the last measured point ($AUC_{0\text{-last}}$) and that extrapolated to infinity ($AUC_{0\text{-}\infty}$) indicated that 6 hours are not sufficient to describe the elimination phase properly. Therefore, another experiment lasting 30 hours was performed. C_{max} of DpC was reached immediately upon the end of *i.v.* administration and the concentration was comparable with that of Bp4eT. Notably, the plasmatic half-life is more than 10 times longer than that of Bp4eT [2] which is beneficial for further development of this compound. The mean residence time (MRT) of DpC is approximately 9 times of that of Bp4eT that might contribute to the prominent efficacy of this compound.

4 Conclusions

In this study, a UHPLC-MS/MS method for quantification of a novel thiosemicarbazone Fe chelating anti-cancer agent – DpC – was developed and fully validated. This method was utilized in a PK study in rats which indicates more favourable PK profile as compared with a previously developed thiosemicarbazone - Bp4eT. The data obtained in this study will be further utilized in the advanced preclinical development of this novel anticancer agent.

Acknowledgement

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P88 SURFACE PLASMON RESONANCE IMAGING - FIRST EXPERIENCE

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1 Introduction

Surface plasmon resonance imaging (SPRi) can be used for the determination of biomolecules on the surface of the chip (prism with nanolayer deposited gold) in real time, without labeling any of the reactants [1]. Analysers applying this technique use CCD camera for sensing of the biochip surface (microscopic image of surface) [2]. Kinetic parameters of these interactions can be also determined [3]. SPRi biosensors are essentially sensitive optical affinity sensors. Biomolecules bound to the surface of the biochip represent so-called biorecognition element which selectively recognizes the determined analyte [4]. The actual SPR phenomenon can be explained this way: the beam of linearly p-polarized light shines on the interface of the prism-metal layer and at an angle it forms an evanescent wave interacting with surface electron plasma. It leads to resonance and creation of a plasmon wave. CCD camera records the decrease in light intensity due to the phenomenon of resonance. Dependence of the reflected light beam intensity on the incident angle is recorded graphically [5].

2 Objectives

The aim of this study was to optimize the preparation of biochips, set up and launch a new SPRi-Lab^{+} ™ device and SPRi-Arrayer spotting equipment (by HORIBA company). For the given task, pilot method for the ovalbuminu (OVA) determination was chosen.

3 Experimental

The so-called CS chip with reactive NHS groups (HORIBA) was applied. The anti-ovalbumin (anti-OVA, monoclonal antibody, clone OVA-14, Sigma-Aldrich), and mouse IgG (m-IgG, negative control, Sigma-Aldrich) in a concentration of 1 g.l^{-1} in 10 mM PBS buffer using SPRi-Arrayer were immobilized on chip. Configuration of antibodies on the chip surface is a checkerboard (36 spots) in diameter 300 μm. For determination of OVA, eight samples of concentrations ranging from 0.001 to 1000

mg.l^{-1} were used, prepared by dilution of a standard OVA (Sigma-Aldrich) in 10 mM of PBS solution. Kinetic curves were obtained for each spot. Data were evaluated in programs SPRiAnalysis and ScrubberGen. Analytical characteristics of the method were statistically calculated from the measured signals of OVA. For processing of calibration curve, software QC Expert 3.0 was used.

4 Results and Discussion

Biochip was spotted with anti-OVA and m-IgG antibodies, SPRi curves were measured and on the basis of inflection points SPR curves, the optimal fixed angle of 58.1° was found for each spot. At this angle, the measurement of calibration samples OVA was realized, Figure 1.

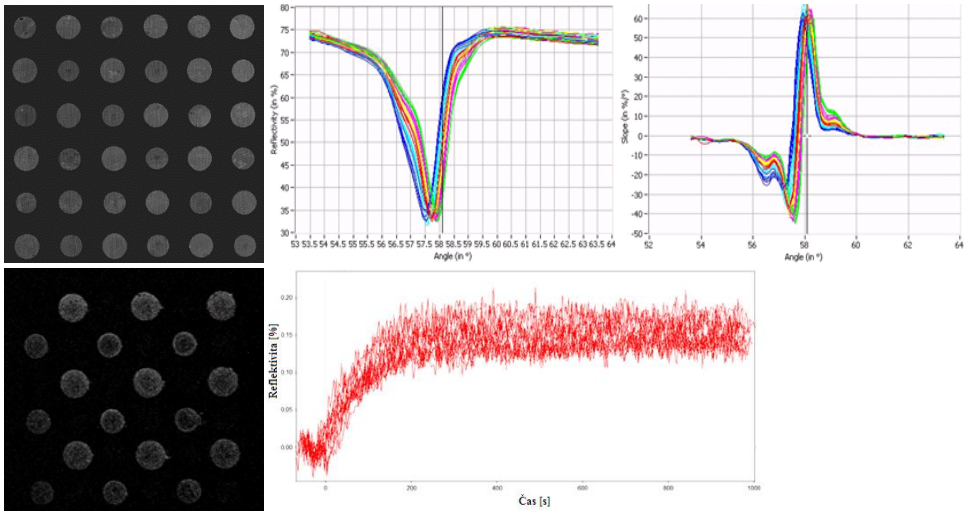

Fig. 1. An example of spot scanning with CCD camera (top left – biochip with immobilized anti-OVA and m-IgG, bottom left - the biochip after interaction with anti-OVA with standard OVA sample at concentration of 10 mg.l^{-1}). Records of SPRi curves (top center) and the calculation of the optimal fixed angle (top right). Sensorgram measurement of OVA at concentration of 10 mg.l^{-1} after correcting signal the m-IgG (bottom right).

The detection capability of the SPRi method determination OVA was tested. The limit of detection based on measurements of the blank was 0.0041 mg.l^{-1}. Below this detection limit the presence of OVA in the sample cannot be reliably detected.

Table 1. The results of measurements of standards OVA and calculated calibration factor.

OVA (mg.l^{-1})	1. signal	2. signal	3. signal	average signal	factor
1000	0.7500	0.7000	0.6800	0.7100	1408.4507
10	0.4400	0.4000	0.4600	0.4333	23.0769
5	0.3300	0.3000	0.3100	0.3133	15.9574
1	0.1000	0.1200	0.1300	0.1167	8.5714
0.5	0.0500	0.0480	0.0530	0.0503	9.9338
0.1	0.0200	0.0240	0.0260	0.0233	4.2857
0.01	0.0100	0.0100	0.0100	0.0100	1.0000
0.001	0.0080	0.0040	0.0065	0.0062	0.1622

The calibration factor (concentration/signal) shows that especially in the start and end point of the curve is the dependence of the signal on the concentration strongly nonlinear. For further calibration dependence processing, these extreme points of calibration curves were therefore removed. By using this method, reliable results at concentration from 0.01 to 10.0 mg.l^{-1} can be provided. For designing the calibration dependence, a quadratic calibration model was used. On the basis of the calibration curve software evaluated parameters listed in Table 2.

Table 2. Parameters of non-linear calibration model of curve for OVA determination.

parameter	estimation of parameter	SD	LL of CI (α = 5 %)	UL of CI (α = 5 %)
a	0.0179	0.0089	-0.0103	0.0461
b	0.0796	0.0071	0.0568	0.1023
c	-0.0038	0.0007	-0.0061	-0.0015

a, b, c = parameters of parabola; LL of CI = the lower limit of the confidence interval; UL = the upper limit of the confidence interval.

Absolute member of the calibration curve is statistically insignificant, the calibration curve passes through the beginning, the absolute term can be neglected, thus the equation of a parabola is: $y = bx + cx^2$. Correlation coefficient of the calibration curve is equal to 0.998, which shows the exact curve-fit. Sensitivity of the calibration curve was calculated, Table 3.

Table 3. Sensitivity of SPRi methods of measurement OVA.

Sensitivity of calibration curve	concentration (mg.l^{-1})
Sensitivity at zero	0.080
Sensitivity at the beginning	0.080
Sensitivity in the middle	0.041
Sensitivity at the end	0.003

Table 4. The calculated calibration limits for the independent variable determining OVA.

Methods	Xc ($mg.l^{-1}$)	Xd ($mg.l^{-1}$)	Xq ($mg.l^{-1}$)
Method by ISO 11843-2	3.646	4.652	7.292
Direct method of analyte	0.472	0.872	1.322
Direct method of signal, IUPAC	0.472	0.887	1.306
Combination method Ebel, Kamm	0.403	0.823	1.264
Method K*Sigma in regresion	0.472	0.968	1.491
Method K*Sigma, ACS	0.384	0.784	1.201

Xc = calibration limit critical, Xd = calibration limit of detection, Xq = calibration limit of quantification.

The limit of detection was determined by six different methodologies, ranging from 0.784 to 4.652 $mg.l^{-1}$, Table 4. The limit of quantification, in which the precision of the determination is expressed as CV of less than 10 %, ranged from 1.201 to 7.292 $mg.l^{-1}$. From the evaluated methodologies for calculating the calibration limits, the most distinguished method of calculation was the one according ISO 11843-2. Other methods provided comparable values of limits because they use the limit of the calibration curve detection for calculation.

4 Conclusions

The preparation of biochips by using the SPRi-Arrayer equipment and determination of ovalbumin were optimized. Analytical characteristics of this method were statistically established. The limit of detection (0.0041 $mg.l^{-1}$) based on measurements of the blank, sensitivity of the method and detection limit of the calibration dependence were determined. Method for the determination of ovalbumin gives reliable results in a concentration ranging from 0.01 to 10.00 $mg.l^{-1}$.

Acknowledgement

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P89 ELECTROLYSIS IN ELECTROMEMBRANE EXTRACTIONS. EFFECTS ON EXTRACTION PERFORMANCE FOR SUBSTITUTED PHENOLS

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Summary
Electrolysis was shown to play a significant role in electromembrane extraction (EME) performance. Alkaline acceptors, prepared as 1 – 10 mM CsOH solutions, suffered from electrolytic reactions during EMEs of substituted phenols and their pH values decreased by more than 8 units after 40 min of EMEs at 50 V. As a consequence, serious deterioration of EME performance was observed for weak and medium-strong acidic phenols due to their compromised ionization and their subsequent back-extraction into organic phase and donor solution. Application of acceptor solutions consisting of high concentrations of weak bases (e.g. 500 mM ethanolamine) ensured similar initial pH conditions as for the alkali-metal hydroxide based solutions, moreover, significantly better tolerance to electrolysis-induced effects was obtained. Stable pH of acceptor solutions was achieved as well as an improved EME performance for all analytes over the entire extraction period.

1 Introduction

EME employs electric field as the driving force for selective transfer of charged analytes from aqueous donor solution across supported liquid membrane into aqueous acceptor solution and offers significant reduction of extraction times in comparison with other micro-extraction techniques. The devices for EMEs are usually down-scaled to be compatible with low volumes of donor and acceptor solutions, which have a direct consequence on their instrumental set-up, i.e. working electrodes are placed directly into the respective solution. The electrode reactions take place in the solutions during EMEs and may have a direct bearing on changes in composition and/or pH value of the solutions, especially of acceptor solution, whose volume is usually about two orders of magnitude lower compared to donor solution. Surprisingly, the effect of electrolysis was not considered or was considered marginal in most previous reports on EMEs and it is the aim of this contribution to describe this phenomena quantitatively.

2 Experimental

2.1 Electromembrane extraction

The EME system was described in a previous publication [1]. The following electrode reactions take place during EMEs:

Anode: $H_2O \rightarrow 2H^+ + ½\ O_2 + 2e^-$
Cathode: $2H^+ + 2e^- \rightarrow H_2$

2.2 Capillary electrophoresis

A 7100 CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with UV-Vis absorbance detector (direct detection at 200 nm) was operated at a potential of + 15 kV applied at the injection side of the separation capillary and was maintained at 25°C. BGE solution consisted of 20 mM $Na_2B_4O_7.10H_2O$ and 10 mM Na_2HPO_4 adjusted to pH 9.8 with 1 M NaOH. Injections were performed at 50 mbar for 5 s.

3 Results and Discussion

Donor solution was prepared as 10 µg/ml of substituted phenols in 0.5 mM KOH (pH 9.4). Various alkaline acceptor solutions (1 mM, 5 mM, 10 mM CsOH and 500 mM ethanolamine) were prepared, which promote EME transfer of acidic phenols into acceptor solutions due to their highly alkaline pH [1]. These solutions have similar pH values (10.9, 11.5, 11.7, 11.8) but differ significantly in concentration and therefore in susceptibility to pH changes due to electrolytic reactions. Figure 1 depicts EME efficiencies for extractions into selected acceptor solutions. Extraction efficiencies varied significantly for the acceptor solutions and the effect of electrolysis was also examined by pH measurements of acceptor solutions after EMEs. The original pH of 1 mM and 10 mM CsOH acceptor solutions dropped-down to 2.0 and 3.7 after 40 min of EME, respectively. As a consequence, decreased recoveries were observed for weak acids (phenol, 4-chlorophenol) and medium-strong acids (pentachlorophenol and 2,6-dinitrophenol) during EMEs. At pH values, which are below their pK_a values (see Fig. 1), the phenols are ionized only partly or not at all and are back-extracted into organic phase/donor solution by diffusion. Extraction efficiencies of picric acid were not affected by pH changes of acceptor solutions due to its strongly acidic character. On the other hand, acceptor solution consisting of 500 mM ethanolamine kept a rather stable pH (ca. 11.8) during the entire 40 min EME and gradual increase in extraction recoveries was observed for all phenols independently of their pK_a values.

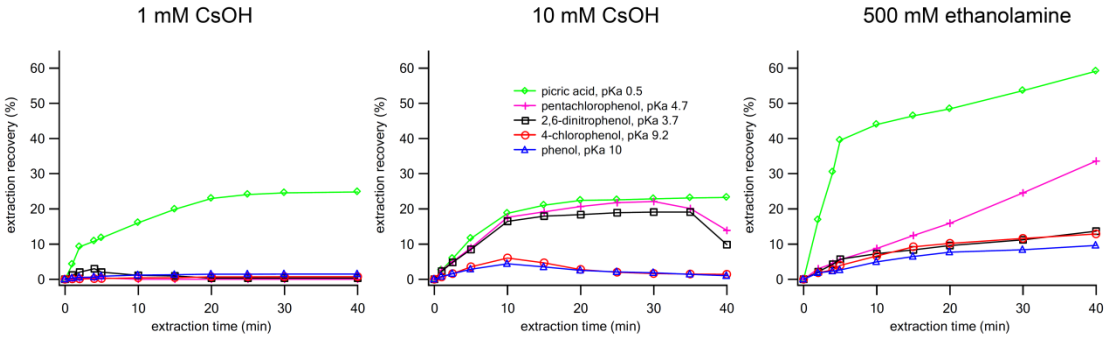

Fig. 1. EME performance for extractions into various acceptor solutions. EME conditions: extraction voltage, 50 V; donor, 10 µg/ml of phenols in 0.5 mM KOH (pH 9.4); SLM, ENB; agitation, 600 rpm.

4 Conclusions

Effects of electrolysis in EMEs were evaluated by time-dependent monitoring of extraction efficiencies for strongly (pK_a = 0.5) to weakly (pK_a = 10) acidic analytes. pH values of alkaline acceptor solutions (prepared from alkali-metal hydroxides) were reduced significantly during 40 min EMEs and resulted into serious deterioration of EME performance for weak and medium-strong acids. A suitable remedy to pH changes of acceptor solutions during EMEs might be application of high concentrations of weak electrolytes, i.e. ethanolamine for EMEs of acidic analytes, which possess the ability to compensate for H^+ formation in acceptor solutions.

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P90 MICRO RNA-124 DETERMINATION EMPLOYING MAGNETIC PARTICLES AND QUANTUM DOTS

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1 Introduction

MicroRNAs (miRNAs) are a novel class of evolutionary conserved single-stranded RNAs, which have an important role in the regulation of gene expression at the posttranscriptional level.

An effect of miRNAs is most often based on the binding to the untranslated region (3'UTR) of target mRNA causing degradation (or inhibition) of target mRNA. It is not surprising that they influence numerous cellular processes such as proliferation, differentiation, apoptosis, metastases, angiogenesis, and immune response [1, 2], of these many are connected with diseases including tumor ones.

Recently, many studies have shown that the miRNAs profile may vary in numerous types of cancers like breast cancer [3, 4], hepatocellular carcinoma [5], lymphoblastic leukemia [6] and prostate cancer [7]. Specific miRNAs also contribute

to neural differentiation of neurons [8], regulate proliferation [9] and gastrulation of stem cells [10].

In this study, we coupled magnetic particles-based extraction with fluorescent labeling by streptavidin-coated CdTe quantum dots and capillary electrophoretic analysis with laser-induced fluorescent detection for detection of miRNA.

2 Experimental

2.1 Magnetic particles-based miRNA isolation

The isolation procedure was carried out according to the scheme shown in Fig. 1A-D. The magnetic microparticles (MPs) Dynabeads M-270 Streptavidin (Life Technologies) were used for miRNA isolation. The biotinylated complementary-DNA probe (5´-Btn-TGG CAT TCA CCG CGT GCC TTA-3´) immobilization on MPs surface was done using 50 µl of MPs and 3 µl of 100 µM biotinylated complementary-DNA probe. The mixture was diluted by water reaching the final volume of 200 µl and incubated for 10 minutes. Further, the hybridization with the target miRNA (5´-UAA GGC ACG CGG UGA AUG CCA-3´) was performed according to Huska et al. [11]. Subsequently, the MPs were resuspended in 50 µl of the elution solution (0.2 M NaCl, 0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4). During the elution, the sample was heated to 70°C for 5 minutes and this caused double-stranded RNA (dsRNA) denaturation and the miRNA was released from MPs surface.

2.2 Conjugation of quantum dots with streptavidin and biotinylated complementary-DNA probe

CdTe QDs capped with mercaptopropionic acid precipitated by isopropanol (0.1 mg/ml in water) were mixed with 10 µl of CDI (10 mM in 100 mM PBS pH=7.4) and the solution was kept at 25°C for 30 min. The mixture was mixed with streptavidin solution (final concentration of streptavidin was 0.02 mg/ml) and the reaction took place at 25°C for 2 hours. Subsequently, streptavidin-coated QDs were conjugated with biotinylated complementary-DNA probe (25°C for 20 min).

2.3 MicroRNA labelling by quantum dots

QDs were conjugated with target miRNA after elution from magnetic particles at 25°C for 30 min.

2.4 Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF)

CE-LIF analysis was carried out using PACE/MDQ instrument (Beckman Coulter). Argon ion laser with wavelength 488 nm was used as an excitation light source and emission was measured at 510 nm. 20 mM sodium borate buffer was used as separation electrolyte and separation voltage was 20 kV. The sample was injected by pressure 1 psi for 5 seconds into the capillary with 75 µm internal diameter, 63 cm total length and 53 cm effective length.

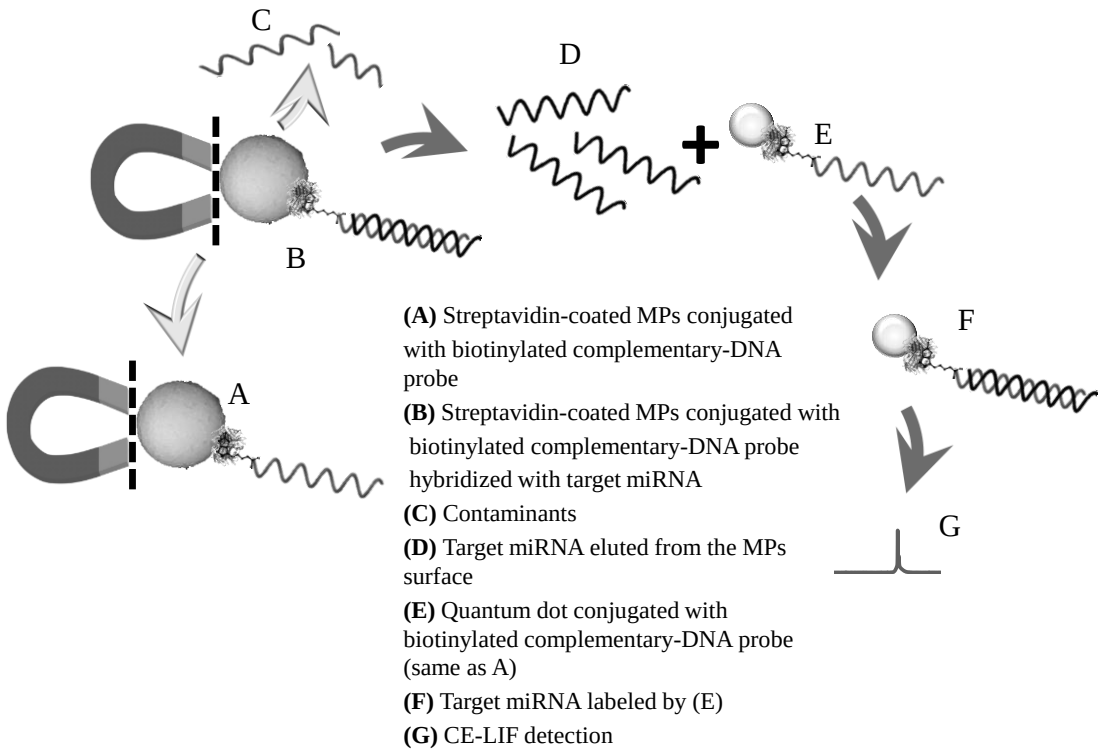

Fig. 1. Scheme of the isolation and detection process.

3 Results and Discussion

The specific isolation of miRNAs by streptavidin-coated MPs was characterized in terms of the yield of extraction. As a first step the ability of the particles to bind the biotinylated complementary-DNA probe was identified and it was found out that 500 μg of MPs bind 1.02 μg of the probe. Moreover, the selectivity of the isolation was characterized by comparison of the isolation yield of target miRNA and three non-complementary fragments of nucleic acid. The results showed approximately 15-times higher signals for the target miRNA.

CE analysis of streptavidin-conjugated QDs showed significant shift in migration time and change in the peak shape when biotinylated complementary-DNA probe was conjugated to the QDs and also further shift after hybridization with the target miRNA which is due to the negative charges of each strand of nucleic acid.

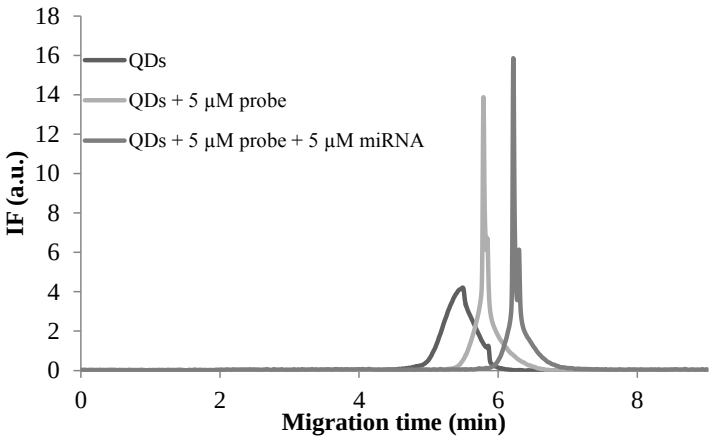

Fig. 2. Electropherograms of QDs, QDs conjugated with specific probe, and QDs with specific probe hybridized with target miRNA.

4 Conclusions

MicroRNAs are showing their potential not only for therapeutic purposes in gene silencing but also for diagnostic applications, therefore their specific and sensitive detection is required. Magnetic particles represent an elegant method for specific isolation and quantum dots provide sensitive option for their optical detection

Acknowledgement

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P91 ZWITTERIONIC MONILITHIC CAPILLARY COLUMNS FOR HYDROPHILIC INTERACTION CHROMATOGRAPHY

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Summary
N,N-dimethyl-N-methacryloxyethyl-N-(3-sulfopropyl) ammonium betaine (MEDSA) as a polar functional monomer in combination with dioxyethylene dimethacrylate (DiEDMA) or bisphenol A glycerolate dimethacrylate (BIGDMA) were used for preparation of capillary monolithic columns suitable for separation of small molecules in hydrophilic interaction chromatography. These columns showed height equivalent to theoretical plate of H = 16.5 μm. Columns provide good preparation repeatability, R.S.D. < 0.7 %.

1 Introduction

Miniaturization is an actual trend in liquid chromatography. A micro-bore or capillary columns allow lower volumes of mobile phases and of samples to be used. One type of columns suitable for use in micro-liquid chromatography are conventional columns filled with spherical particles that can be totally porous, with porous layer or non-porous. The particle size is usually about 1 to 5 μm. Another type of chromatographic columns are columns filled with monolithic stationary phases. Monolith is formed by a single piece of highly porous material which contains flow through pores and limited populations of mesopores. Monolithic stationary phases may be either inorganic monolithic stationary phases [1, 2, 3] or organic polymers. Organic polymers can be divided into several groups: acrylamide-based monoliths [4, 5], styrenes [6, 7], and methacrylate-based organic monoliths [8, 9, 10, 11].

Organic polymethacrylate monoliths are prepared by in situ radical polymerization of polymerization mixture in fused silica capillary, containing crosslinking monomer, functional monomer, porogenic solvents, and thermal initiator of polymerization reaction.

2 Experimental

Dioxyethylene dimethacrylate (DiEDMA), bisphenol A glycerolate dimethacrylate (BIGDMA), N,N-dimethyl-N-methacryloxyethyl-N-(3-sulfopropyl) ammonium betaine (MEDSA), l-propanol and 1,4-butanediol, azobisizobutyronitrile were obtained from Sigma-Aldrich, Steinheim, Germany. Distilled water was purified in a DEMIWA 5ROI station, Watek, Ledeč nad Sázavou, Czech Republic. Polyimide-coated fused-silica capillaries with 320μm internal diameter, were purchased from J&W, Folsom, CA, USA. To improve the stability of the polymethacrylate monolith

bed inside the silica capillary, the inner wall of capillary was modified by 3-(trimethoxysilyl) propyl methacrylate.

3 Results and Discussion

It has been determined the effect of the applied crosslinking monomers on the efficiency of prepared zwitterionic monolithic columns using van Deemter plots. The column efficiency was expressed in terms of height equivalent of theoretical plate (HETP, µm) for toluene measured in 95% acetonitrile at linear mobile phase velocity of 0.5 mm/s. The dioxyethylene dimethacrylate and bisphenol A glycerolate dimethacrylate columns showed approximately 70 000 theoretical plates/m for toluene at minimum of van Deemter plot (H = 16.5 µm).

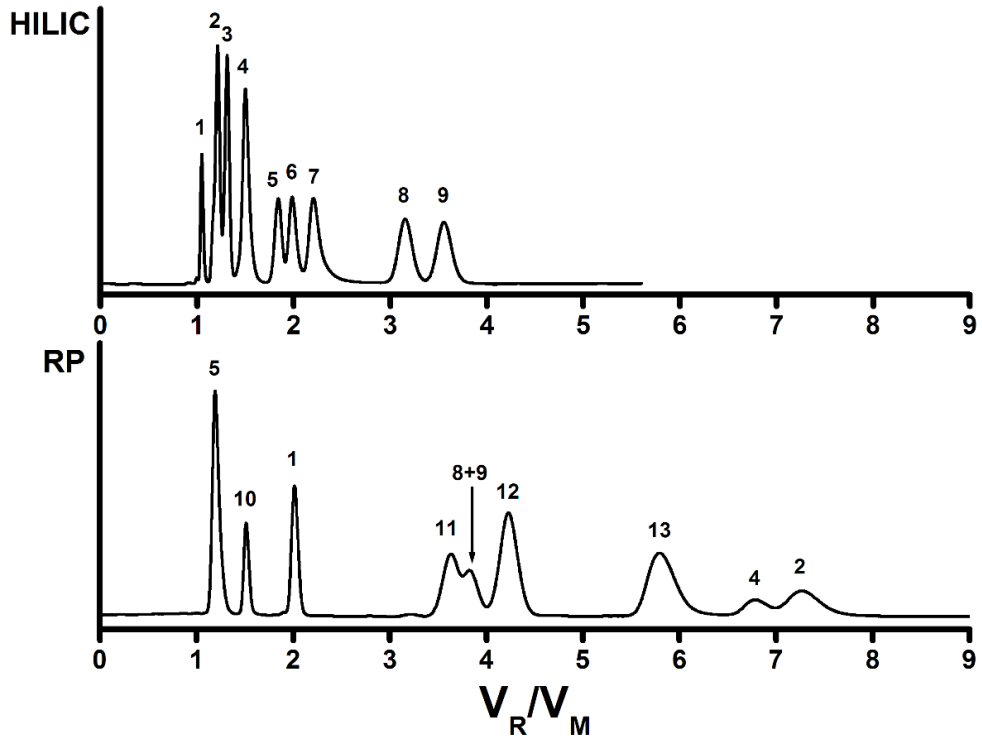

Fig. 1. Separation of flavones in HILIC and RP mode on BIGDMA column. HILIC: 80% ACN/ 20% 10 mM NH_4Ac in water (pH 3), F_m = 2.1 µl/min, p = 1.7 MPa; RP: 40% ACN/60% 10 mM NH_4Ac in water (pH 3), F_m = 3.1 µl/min, p = 4.4 MPa, UV detection at 214nm, l = 123mm, i.d. 320 µm. Analytes: 1 – vanillin, 2 – biochanin A, 3 - 7 – hydroxyflavone, 4 – apigenine, 5 – esculine, 6 – quercetine, 7 – morin, 8 – (+)-catechine, 9 – (-)-epicatechine, 10 – naringine, 11 – 4-hydroxycoumarine, 12 – hesperetine, 13 – naringenine.

To check the elution properties of dioxyethylene dimethacrylate and bisphenol A glycerolate dimethacrylate columns, the repeatability was evaluated using a test mixture containing toluene, phenol, uracil, and thiourea in 95% acetonitrile as a mobile phase. The relative standard deviations of retention volumes were lower than

0.7% for all types of columns. The bisphenol A glycerolate dimethacrylate column shows dual retention mechanism, which allows separation both in HILIC and RP mode (Fig. 1). HILIC separation provides more symmetrical peaks with lower retention in comparison with reversed mode. In addition, HILIC mode allows baseline separation of diastereoisomers (+)-catechine and (-)-epicatechine.

4 Conclusions

The crosslinking monomer in the polymerization mixture significantly affects chromatographic efficiency. Capillary monolithic columns prepared by using dioxyethylene dimethacrylate and bisphenol A glycerolate dimethacrylate provide efficiency up to 70 000 plates per meter. These columns have excellent preparation repeatability and long stability. Optimized columns were used for isocratic separation of polar compounds both in hydrophilic interaction and reversed-phase modes.

Acknowledgement

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P92 THE INFLUENCE OF STORAGE ON THE SENSORY QUALITY OF PROCESSED CHEESE ANALOGUES

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Summary
Flavour of four types of processed cheese analogues with different vegetable fats (apricot, flaxseed, grape, black currant) was evaluated during several months of storage using sensory evaluation. The influence of fat used on flavour and overall sensory quality of samples was also followed. The seven-point hedonic scale was used for sensory evaluation of appearance and colour, gloss, texture and taste and aroma (flavour) of samples. The intensity of selected descriptors expressing off-flavour (e.g. sour, oily, bitter, salty, cheesy and others) was then evaluated using profile test.

1 Introduction

Cheese and processed cheese analogues are the new trend in the dairy industry. During their production milk fat, milk protein or both are partially or wholly replaced by non-milk components, principally of vegetable origin. Lower raw material costs are the main reason for their production, however, these products also offer a number of positive nutritional aspects, e. g. higher proportion of unsaturated fatty acids, less or no cholesterol, reduced amounts of saturated fat and sodium, lower calorie content. Due to these nutritional benefits they are becoming the sought food [1, 2]. Sensory properties of processed cheese analogues are influenced by many factors including raw material (type of natural cheese, the lactose content and melting salts, the kind of vegetable oil), the melting process and/or the storage conditions [3, 4, 5]. Replacing milk fat with vegetable oils is accompanied by typical textural defects, deterioration of melting properties and formation of insufficient flavor, but this risk can be minimized by suitable choice of materials and production conditions. Just flavour, which is defined as a combination of taste, aroma and mechanical stimuli in the oral cavity during eating and often is the determining factor for the consumer acceptability of cheese [6], is the main weakness of analogues, being less distinctive than for conventional products [1, 2].

The aim of this work was to evaluate flavour of processed cheese analogues with different vegetable fats during several months of storage using sensory evaluation.

2 Experimental

The main raw materials for the production of processed cheese analogues (40% (w/w) of dry matter, 50% (w/w) fat in dry matter) were: Edam cheese (30% (w/w) fat in dry matter), maturity of 8 weeks (Kromilk, a.s.), butter (~ 82% (w/w) solids, ~ 80% (w/w) fat in dry matter), various oils (apricot, flaxseed, grape and black currant), melting salts (2.5% (w/w)) and water. The samples were evaluated immediately after manufacture and then after 3 months of storage. Students of FCH BUT in Brno were selected and trained for sensory evaluation. Selected sensory characteristics (appearance and color, gloss, texture and flavor) were evaluated using seven-point category ordinal hedonic scale (1 unacceptable ⇒ 7 excellent) (ISO 4121); selected taste descriptors (sour, salty, bitter, cheesy, oily, other pleasant and/or unpleasant taste) were evaluated using profile test (1 imperceptible ⇒ 7 very strong) (EN ISO 13299). Pure water and white bread were used as taste neutralizer. . The results were statistically analyzed by the Kruskall-Wallis test ($\alpha = 0.05$) using Unistat software version 5.5.

3 Results and Discussion

Sensory properties, considered by consumers as the main part of overall quality in most processed cheeses, were selected as the indicators of cheese matrix changes during storage. The samples evaluated after 3 months storage showed a better appearance and color, gloss and texture than immediately after production. Among them analogue with black currant oil had the worst evaluation in these parameters. No changes were detected in taste and flavour. The flavour of samples with flaxseed and grape oils was evaluated like insufficient, on the other side analogue with apricot oil was rated as excellent.

The results of profile test are expressed in Fig.1. As can be seen, the greatest changes were noted in analogues with flaxseed oil. These samples were more salty, bitter and oily after storage, also showed more intensive unpleasant taste, described by evaluators as artificial. In the samples with oil from black currant no changes were found during storage experiment, except bitter taste, which was more intensive after storage. Small differences were recorded during storage when evaluating samples with apricot and grape seed oil, however, analogues with apricot oil showed strong intensive cheesy and pleasant taste in contrast to very strong and unpleasant taste in samples with grape seed oil.

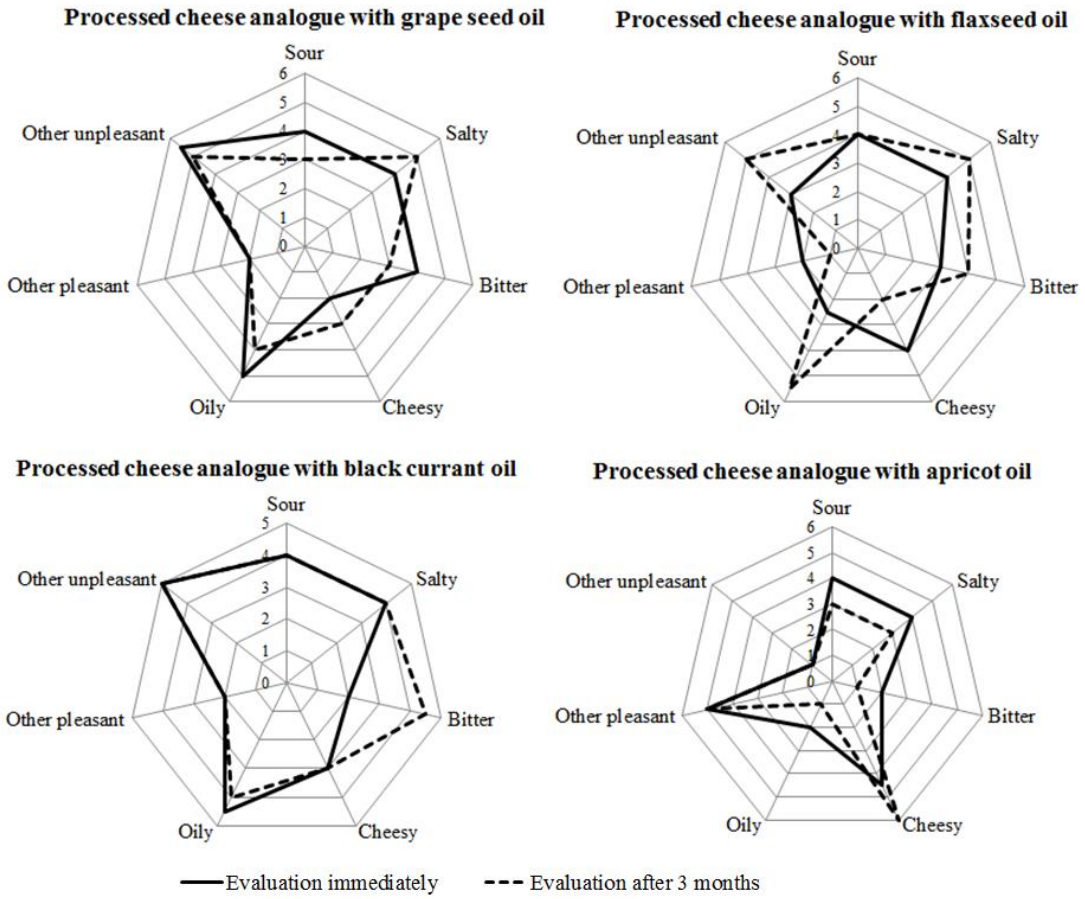

Fig. 1. The intensity profile of selected taste descriptors of processed cheese analogues.

4 Conclusions

Storage experiment revealed that oil used has higher influence on final form of analogue than a few months storage. Grape and black currant oil appears to be rather unsuitable for production of processed cheese analogues due to the negative sensory properties like unpleasant oily, musty and/or artificial off-flavor, unlike apricot oil, whose sensory quality is comparable with classic processed cheese.

Acknowledgement

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P93 STABILITY OF PARATHYROID HORMONE IN HUMAN WHOLE BLOOD

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1 Introduction

Parathyroid hormone, parathyrin (PTH) is an 84-amino acid polypeptide produced by parathyroid glands and released in response to extracellular ionized calcium level. PTH raises serum Ca^{2+} provoking its release from bone by the stimulation of osteoclastic activity, its reabsorption in the kidney (while phosphorus excretion is increased), as well as increase in intestinal absorption through increased calcitriol formation in the kidney. Biologically active PTH circulates as an 84 amino acid peptide and is metabolized within minutes to N-terminal, C-terminal and mid-regional fragments of varied lengths [1]. It was shown that kidneys are responsible for the disposal of small and large C-terminal PTH fragments [2] because in terminal renal failure, both small and large C-terminal PTH fragments represent more than 95% of circulating PTH in circulation, leaving less than 5% PTH (1–84) [3]. Moreover, PTH is also unstable in vitro after phlebotomy and the results of PTH concentrations are very dependent on the method of determination [4] and the type of tubes used for blood collection [5]. We have recently validated the biointact PTH (1-84), a third generation of PTH assay kit. This kit is only able to determine the whole 1-84 PTH, not C-terminal fragments which accumulate in patients with impaired renal function. The aim of our study was thus to evaluate the stability of the (1–84) PTH assayed with the 3rd generation PTH on Cobas E411 at different temperatures and with different sampling tubes.

2 Experimental

We collected three different tubes of fresh blood from each of 10 patients who were undergoing hemodialysis at the Clinic of Internal Medicine of the University Hospital Ostrava. The samples were drawn into 9 mL S-Monovette® plastic tubes containing 14.4 mg of K3-EDTA, 9 mL S-Monovette® plastic tubes containing144 I.U. of lithium heparin and 9 mL S-Monovette® plastic tubes containing clotting activator (Sarstedt AG & Co., Germany). Twenty minutes later all collected samples were separated into seven whole blood aliquots, with the exception of serum samples. Serum samples were aliquoted after 20 minutes of clotting followed by centrifugation (2000g, 4°C, 10 min.). One out of seven aliquots from all type tubes was immediately spun (2000g, 4°C, 10 min.) and PTH concentration was assayed. Obtained results constituted the 'zero-point' (T0). The other aliquots were kept as the whole blood or serum at room temperature (RT) or +4 °C. After 2 (T2), 6 (T6) and 24 hours (T24) of incubation, the samples were centrifuged and PTH concentration assayed. The PTH concentrations were measured according to the manufacturers' instructions using the following commercially available immunoassay: the COBAS Elecsys® PTH(1–84) immunoassay (Roche Diagnostics, GmbH, Germany) on Cobas e411. All the statistical tests were evaluated at the significance level of 5 %. The Shapiro-Wilk test was used to test the normality of the data. The effect of time and temperature on PTH concentrations was tested using the Two-Way Analysis of Variance. These analyses were performed using R software.

3 Results and Discussion

We measured the stability of the (1–84) PTH in the whole blood during a storage at different temperatures and with different sampling tubes. At T0, the Wilcoxon test showed no significant difference among EDTA plasma, lithium heparin plasma and serum (median: 24.41 vs. 21.16 vs. 23.96 pmol/L, respectively; $p>0.05$). Figure 1 shows PTH concentrations observed in the whole EDTA blood: PTH was found to be stable 24 h at both RT and +4 °C (decrease was not greater than 10%). In the whole lithium heparin blood, PTH was stable 6 h at RT and 24 h at +4 °C. In serum, PTH was stable up to 2 h at RT and 24 h at +4 °C. More rapid PTH degradation in serum type tube could be explained by the activity of endogenous human thrombin, which is activated during clot formation [5]. Surprisingly, all observed decrease in PTH levels in different types of tubes was not significant (Table 1).

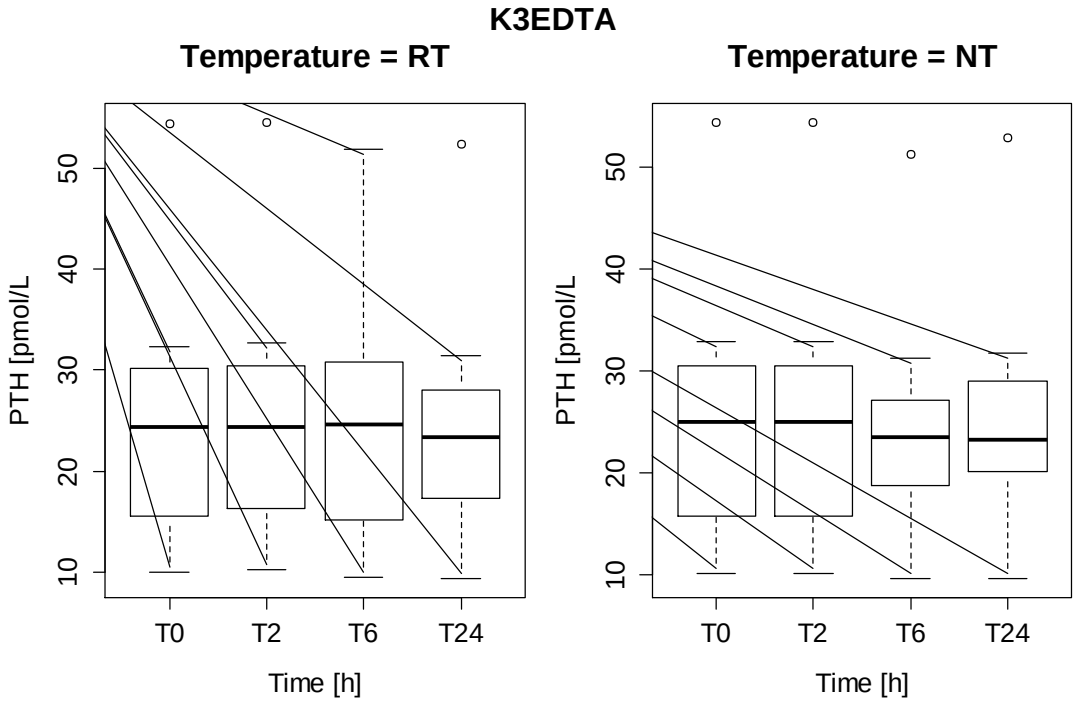

Fig. 1. Changes of PTH concentration in EDTA whole blood at two temperatures.

Table 1. Percentage changes of PTH concentration at different temperatures and with different sampling tubes.

Material	Temperature	T2	T6	T24	Significance
EDTA whole blood	RT	0.14	0.90	-4.28	n.s.
	4°C	2.13	-3.93	-4.73	n.s.
Lithium heparin blood	RT	-6.74	-4.22	-18.89	n.s.
	4°C	-1.17	1.13	-4.15	n.s.
Serum	RT	-6.60	-14.36	-35.89	n.s.
	4°C	-3.52	-2.44	-11.84	n.s.

n.s. means not significant

Finally, our results show that (1–84) PTH (3rd generation kit) is more stable, which could have been expected based on the results previously obtained with 'intact' PTH assays (2nd generation kit) [4]. However, Hocher B. suggested a need of the fourth generation kit because in patients with oxidative stress (dialysis patients) PTH is oxidized at methionine residues at position 8 and 18 [6]. Since oxidized PTH is biologically inactive, currently used method to detect PTH in daily clinical practice may not adequately reflect PTH-related bone and cardiovascular abnormalities in patients on dialysis.

4 Conclusions

In conclusion, greater stability of PTH was found when blood was collected to EDTA type tubes, even in the case of storage at room temperature. Greater stability of PTH measured by the 3rd generation kit assay is one of the very first advantages to move from ‘intact’ to 3rd generation kits.

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P94 PREANALYTIC PHASE OPTIMIZATION OF ETHYL FERULATE ANALYSIS AFTER PENETRATION EXPERIMENTS INTO THE SKIN WITHIN VARIOUS DELIVERY SYSTEMS

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Summary

This study deals with optimization of ethyl ferulate (EF) after skin penetration tests. The skin is known to be difficult biological matrix to deal with. Therefore, sample preparation was crucial part in this case. We found that EF has been degraded during skin penetration tests which was most likely due to resident bacterial microflora. Optimization of antimicrobial content and extraction point solved low recovery values. Furthermore, we confirmed that polymeric micelles based on hyaluronic acid improved penetration abilities of EF.

1 Introduction

EF, an ester of ferulic acid, is common cosmetic agent with highly hydrophobic properties which hinder its delivery into the skin. For this purpose, delivery systems

based on hyaluronic acid has been developed to improve penetration abilities of hydrophobic substances [1]. Recently, skin penetration characteristics for individual cosmetic compounds has begun to be strongly required by cosmetic industry. Analysis of EF levels was done by standardized procedure. However, presence of EF in polymeric micelles and skin as complex biological matrix required specific approach for sample preparation. The aim of our study was to evaluate penetration abilities of EF in various delivery systems and optimize method to gain substantially sufficient values of recovery.

2 Experimental

Penetration experiments with excised porcine ear skin were done using Franz-type diffusion cells for 24 hours under 37 °C. Tested substance was added into donor phase of diffusion cell atop of the skin and after 24 hours donor fluid, epidermis, dermis and receptor fluid has been sampled and processed. We used isopropanol or ethyl acetate as extraction solvent. EF were assessed by HPLC-UV/VIS using Waters Spherisorb C6 column at 30 °C and flow rate 1 $ml.min^{-1}$. Mobile phase consisted of 0.1 % formic acid and methanol with change in ratio during gradient elution. EF was detected by poly diode array detector at 325 nm.

3 Results and Discussion

We found that isopropanol which was regularly used for most penetration experiments was not suitable for extraction of EF from the skin. Ethyl acetate seemed to be better option as recovery had higher values. However, further analysis revealed presence of impurities that were identified as breakdown products of EF. Breakdown of EF could be either result of tough environment conditions, persistent activity of skin enzymes or resident bacterial microflora. We found out that breakdown of EF was probably due to bacterial contamination. After addition of antimicrobial content presence of impurities significantly decreased and recovery of EF reached sufficient values. Some of the papers are also describing bacterial strains that are able to degrade EF [2, 3]. Furthermore, it was found that polymeric micelles based on hyaluronic acid increased the penetration of EF into the epidermis and dermis 24-h post-application in comparison to pure EF.

4 Conclusion

The skin represent very tough biological matrix which is tricky for convenient sample preparations. We managed to optimize EF analysis after skin penetration tests into sufficient scale. This method will no doubt be further upgraded. Moreover, our results confirm that our delivery system based on hyaluronic acid is able to improve penetration abilities of EF, hence penetration abilities of hydrophobic substances.

Acknowledgements

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P95 RARE CELL CAPTURE DEVICE OPERATING WITH CD44 ANTIBODY FUNCTIONALIZED MAGNETIC BEADS

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Summary
A magnetic bead based microfluidic cell capture device was designed, prototyped and tested in this study. The microfluidic environment was designed as a high performance alternative for commercially available systems, manufactured in-house by 3D printing. CD44 antibody functionalized magnetic beads were used as affinity agents. Different sized magnetic beads were applied to investigate the robustness of the system. Cell capture performance was tested using a challenging model system of mouse blood and HT29 human tumor cell mixture. The proposed device showed excellent selectivity for the target cells with high yield.

1 Introduction

Rare cell capture is a rapidly emerging sample preparation method in the biomedical field [1-2]. Microfabricated cell capture devices (MCCDs) overcome the common drawbacks of traditional sell sorters [1] and represent a promising avenue for future developments. Such drawbacks are the requirement for pre-sorting (filtering, centrifugation and rinsing), long sorting time and the requirement of large sample volumes. Furthermore, conventional devices need highly trained service personnel. MCCDs can be used for sorting, processing and analyzing limited amount of biological samples, such as rare cells [3]. Capturing and analyzing circulating tumor cells (CTCs) has major importance in cancer prevention and therapy. Hence, cell sorting has particular importance in cancer research since it reduces the complexity of the biological sample (blood) for liquid biopsy [4].

Currently, the commercially available microfluidic based cell capture devices are very complex and expensive systems requiring specially trained operators. Affinity based cell capture techniques use antibodies [5]. The applied antibodies can be immobilized on the surface of microchips or reaction chambers can be filled with antibody coated magnetic beads [6]. We report on the development and application of a simple, low cost, in-house made MCCD device with the use of CD44 antibody functionalized magnetic beads. Magnetic bead technique is more flexible since particles can be manipulated (retained in flow) with external magnetic field according to special needs.

2 Experimental

The microfluidic device was designed in CAD environment (freeCAD 0.14) and further prototyped by 3D printing (3DFactories, Brno) using polylactic acid (PLA) as thermoplastic. The system comprised a multi-purpose chip holder, a magnet moving assembly and a driving module (Figure 1). A strong neodymium magnet (N348; 1.37-1.42 T; 40 mm x 10 mm x 5 mm, Euromagnet) was placed directly under the microfluidic chamber to ensure sufficient drag force for the various size magnetic beads. The transmission slowed down the 6 rpm motor speed to 1 rpm in order to stabilize the hydrodynamics of the fluid bed. Hydrophilized rombic chip (32.5 mm x 6.5 mm x 0.5 mm, 120 µl) was used as reaction chamber made of cyclic olefin copolymer (microfluidic ChipShop, Jena, Germany). The cell capture performance of the developed device was tested using a challenging model system of mice blood and human tumor cell mixture. The samples were injected with kdScientific (Holliston, USA) syringe pump using 500 µl Hamilton syringe (Bonaduz, Switzerland). The flow rate was set to 1500 $\mu l \cdot h^{-1}$ to avoid any possible mechanical damage of the cells. The chamber was washed and pretreated before each experiment with 2 $mg \cdot ml^{-1}$ BSA in PBS (1X) for 15 min to reduce any nonspecific binding of the target cells.

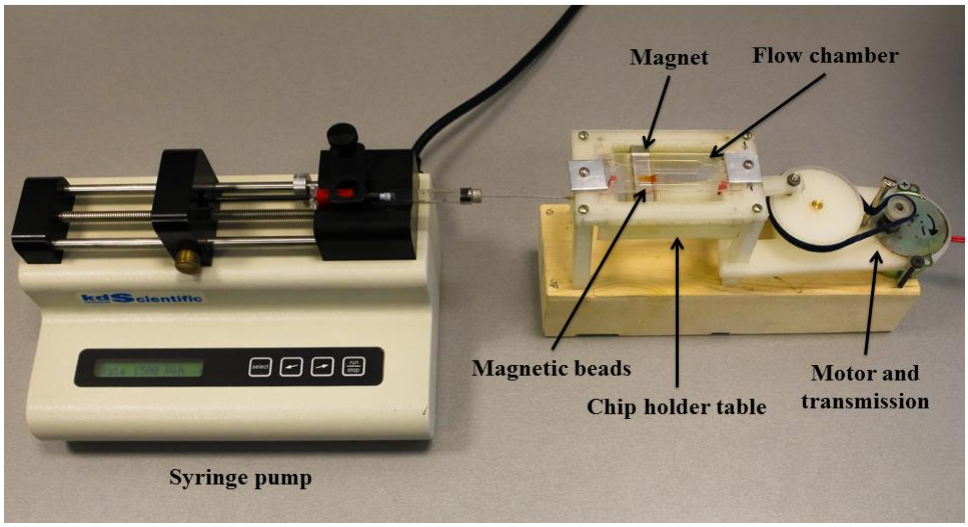

Fig. 1. Experiment setup for CTC capture.

The HT29 colon cancer cell line were used kindly provided by the National Institute of Oncology (Budapest, Hungary). The cells were kept at 37 °C in 5% CO_2 in vented cell culture flasks and handled under sterile condition. 50 µl of mouse blood was used with 450 µl BD FACS (New Jersey, USA) lysing solution that lysate the erythrocytes thus preventing the channels from clogging. Approximately 10,000 cells were used in the model system.

For affinity based CTC capture, CD44 antibody (200 $\mu g \cdot ml^{-1}$, Beckmann Coulter) was immobilized oriented onto 1 µm Chemicell (Berlin, Germany) and 4 µm Spherotech (Lake Forest, CA, USA) diameter Protein A coated magnetic beads by means of an improved binding protocol. The concentration of the beads were 10 $mg \cdot ml^{-1}$ in both instances. As a first step of the coating procedure, 200 µl Protein A magnetic bead suspension was mixed with 40 µl antibody solution. After overnight incubation at 4°C, 30 mM dimethylpimelimidate (DMP) and 30 mM dimethylsuberimidate (DMS) crosslinking reagents were applied. After the crosslinking reaction, the magnetic beads were washed in 150 mM ethanolamine in 0.1 M borate buffer (pH 9), than three times in borate buffer (0.1 M, pH 8.2). Finally, the beads were resuspended in 200 µl 0.1 M borate buffer storage solution (pH 8.2) containing 0.02% sodium-azide (see steps in Figure 2) [7-8].

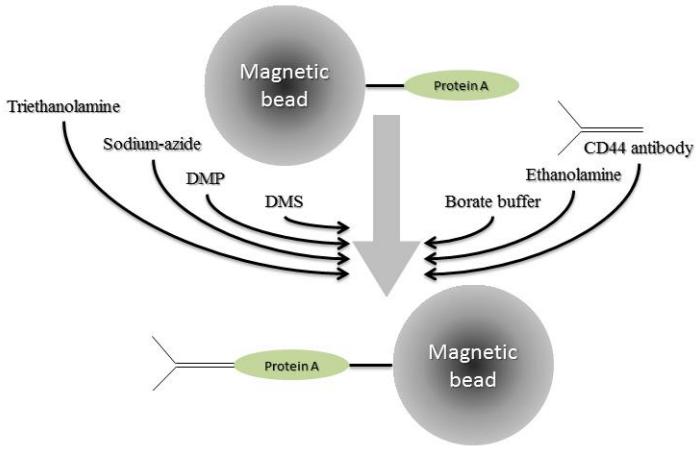

Fig. 2 Antibody binding process to magnetic beads.

Cell capture efficiency was investigated by means of an Eclipse E100 light microscope (Nikon, Tokyo, Japan) equipped with standard Bürker-chamber. Cells were counted from representative samples of the effluent.

3 Results and Discussion

Two sizes of CD44 functionalized magnetic beads were investigated to capture colon tumor cells from blood sample. As shown in Figure 3, both sizes resulted in high efficiency capture from the sample. However, not every cell of interest were captured by the magnetic beads. Some larger cells, mostly HT29 colon cancer cells were physically filtered by the consistently moving magnetic bead bad. The strength of the binding was adequate to retain the cells in the reaction chamber in the flowing system.

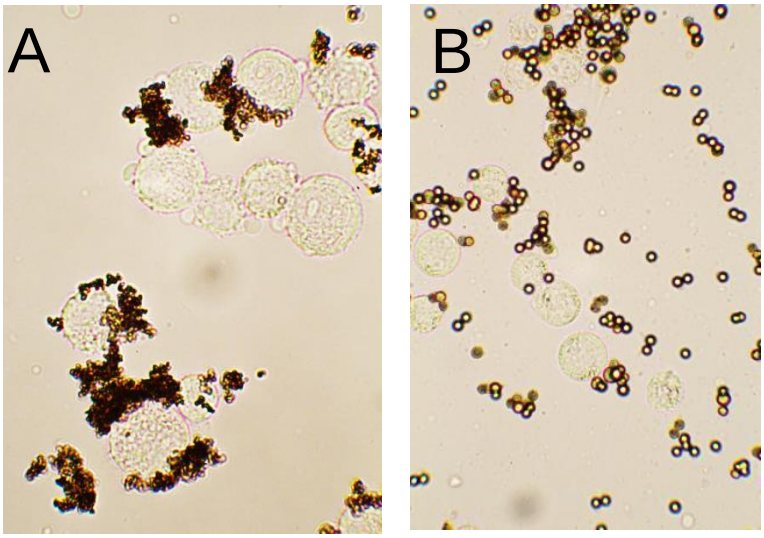

Fig. 3 The captured cancer cells with 1 µm (A) and 4 µm beads (B).

From the effluent, after 10 repeated sampling no cells were counted in the Bürker chamber proving outstanding cell capture yield. However, the morphology analysis of the samples showed some damaged cells that was most likely caused by the moving magnetic beads.

4 Conclusions

This new design of a magnetic based cell capture device reported here showed highly efficient capturing capability using various magnetic particle sizes. No cancer cells were observed in the effluent during numerous cell capture experiments. The improved protocol of antibody binding to Protein A coated beads proved to be capable to capture and hold the cancer cells within the flow chamber at the optimal flow rate of 1500 $\mu l \cdot h^{-1}$.

Acknowledgement

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P96 ANALYSIS OF PHARMACEUTICAL ADDITIVES BY MICROCHIP ELECTROPHORESIS – SURFACE ENHANCED RAMAN SPECTROSCOPY

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Summary
A new and original analytical approach for the analysis of pharmaceutical additives - four synthetic dyes by isotachophoresis (ITP) on a microchip combined with surface enhanced Raman spectroscopy (SERS) detection is described. A channel-coupling (CC) microchip was used for ITP separation of Ponceau 4R, Brilliant black, Sunset Yellow and Azorubine with the aid of properly selected discrete spacers. SERS detection was performed in the second separation channel of the CC microchip containing silver colloidal solution. Proposed ITP-SERS approach allowed reliable identification of studied dyes up to 2.10^{-7} M concentration present in the model samples. Finally, ITP-SERS performed on the microchip was evaluated for analyses of dyes in various pharmaceutical products.

1 Introduction

Permanent progress in the field of analytical chemistry has given a rise to a new analytical instrumentation. Nowadays, several new detection techniques are being developed in the field of microchip electrophoresis (MCE). Few methods were used for detection in MCE, such as laser inducted fluorescence [1], electrochemical detection [2], mass spectrometry [3], but also nuclear magnetic resonance, infrared spectroscopy and Raman spectroscopy [4]. Walker et al. carried out ITP separations of the herbicides on a glass microchip by normal Raman spectroscopy [5]. Although surface enhanced Raman spectroscopy (SERS) detection coupled with MCE has not been published yet, a combination of SERS detection with conventional capillary electrophoresis was successfully developed [6].
This work is dealt with an implementation of unconventional spectral detection technique – SERS for the MCE, when ITP was preferred for the separation of synthetic dyes on the channel-coupling (CC) microchip.

2 Experimental

A poly(methylmethacrylate) (PMMA) microchip with the CC technology and with integrated conductivity detection sensors was used for ITP separations. Raman spectra

were obtained using a Raman Spectrometer B&W Tek Model BWS 415-532H. Silver colloidal solution was prepared by Lee-Meisel method [7]. Stock solutions of synthetic dyes (Ponceau 4R, Brilliant Black, Sunset Yellow and Azorubine) and discrete spacers (glutaric, acetic, butyric, valeric and pantothenic acids) were prepared at 10 mM concentrations from chemicals of analytical grade (Sigma-Aldrich and Merck Millipore). Chemicals of analytical grade (Sigma-Aldrich) were used for the preparation of leading (LE) and terminating electrolyte (TE) solutions. The two pharmaceutical samples (Coldrex and Strepsils) analyzed in the study were purchased in local pharmacy. Sample pretreatment included only dissolution, 2 min centrifugation and appropriate dilution.

3 Results and Discussion

Electrolyte solutions with pH 6.0 (LE) and 6.1 (TE) were used for ITP separations of synthetic dyes. Satisfactory resolution of all studied analytes was achieved by adding β-cyclodextrin to the LE solution. Discrete spacers were added to the samples in order to achieve a spatial distribution of dyes. The conductivity detection sensor was implemented at the end of the first separation channel of the CC microchip. An isotachopherograms of studied analytes were obtained from this detector (Fig. 1 A,B). ITP separations continued in the second separation channel of the CC microchip, which was filled with LE and silver colloidal solution in 1:1 ratio. Laser for Raman spectroscopy was focused on the beginning of the second separation channel of the CC microchip. Raman spectra for analytes were acquired in timeline of 50 ms during ITP separation process. The lowest injected concentrations of synthetic dyes, at which Raman spectra were satisfactory acquired, were 2.10^{-7} M (Fig. 1 B1*-B4*).

This new microanalytical approach proposed for the analysis of synthetic dyes was applied to two pharmaceutical products (Coldrex and Strepsils). The samples were dissolved, centrifuged, diluted and injected in the presence of discrete spacers to the CC microchip. Synthetic dyes Ponceau 4R and Brilliant Black were reliably identified in Coldrex and Ponceau 4R and Sunset Yellow were identified in Strepsils by ITP-SERS analyses on the CC microchip.

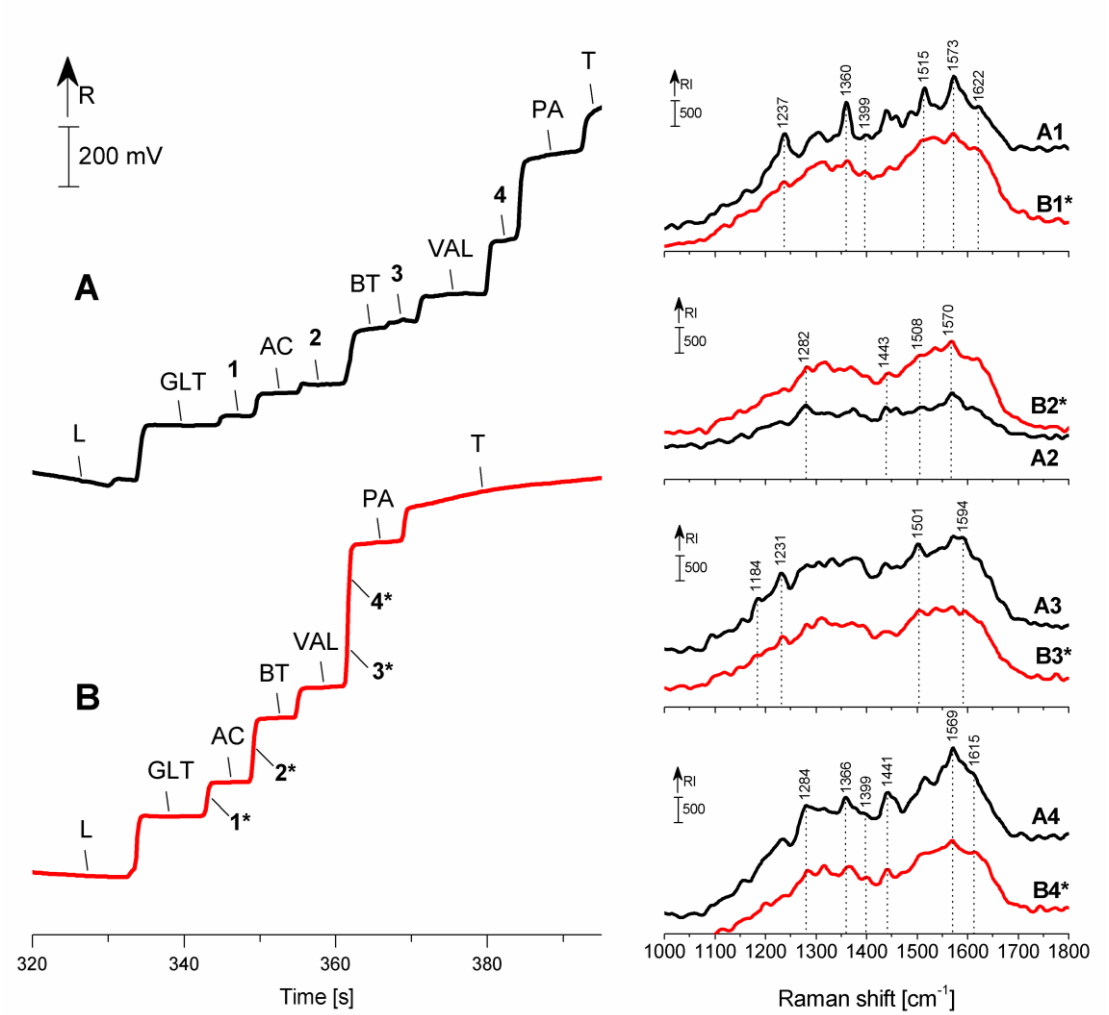

Fig. 1. ITP-SERS analyses of synthetic dyes (1; 1* = Ponceau 4R, 2; 2* = Brilliant Black; 3; 3* = Sunset Yellow; 4; 4* = Azorubine) on the CC microchip. ITP separations of synthetic dyes (A) at 0.2 mM concentrations and (B) at 0.2 µM concentrations with addition of discrete spacers (GLT = glutarate; AC = acetate; BT = butyrate; VAL = valerate; PAN = pantothenate) at 0.5 mM concentrations. SERS spectra of (A1, B1*) Ponceau 4R, (A2, B2*) Brilliant Black, (A3, B3*) Sunset Yellow and (A4, B4*) Azorubine acquired under the given ITP separation conditions. L = leading ion; T = terminating ion; R = resistance; RI = Raman intensity.

4 Conclusions

This study has clearly showed new possibilities for unconventional spectral detection in the MCE. SERS has enabled reliable identification of studied synthetic dyes after ITP separation on the CC microchip. Proposed ITP-SERS approach has been successfully applied to the analyses of various pharmaceutical products.

Acknowledgement

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P97 RAPID DETERMINATION OF METFORMIN, PHENFORMIN AND PROPYL TRIPHENYLPHOSPHONIUM BROMIDE IN MITOCHONDRIA MATRIX BY CAPILLARY ELECTROPHORESIS

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Summary

Capillary electrophoretic method with contactless conductivity detection have been developed for determining the oral antidiabetic drugs metformin and phenformin in mitochondria matrix that was isolated from the skeletal muscles of laboratory rats.

1 Introduction

Metformin and phenformin (Fig. 1) are oral antidiabetic drugs of the biguanide group. At the present time, metformin is the drug of choice for treating type II diabetes and is prescribed primarily for overweight people. Clinical studies have demonstrated that ten-year administration of metformin to obese diabetics reduces morbidity and mortality by 30 % compared to treatment with insulin and sulphonyl urea. In addition, administration of metformin to obese and overweight diabetics does not contribute to a further increase in their weight. Metformin is further characterised by a low risk of lactic acidosis compared to other biguanides. At the present time, metformin is the most extensively used antidiabetic drug and 48 million packages were prescribed in 2010 in the U.S.A. alone. This study is focussed on the determination of metformin and phenformin in the mitochondria matrix to reveal the entrance of these drugs into mitochondria.

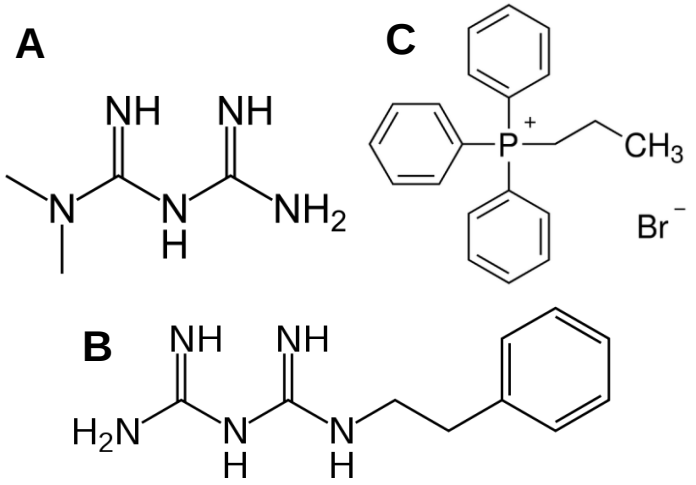

Fig. 1. Metformin (A), phenformin (B), propyl triphenylphosphonium bromide (C).

2 Experimental

Electrophoretic measurements were carried out using the HP^{3D}CE system (Agilent Technologies, Waldbronn Germany) equipped with a contactless conductivity detector (C^{4}D), which is placed in the electrophoretic cassette at constant temperature of 25 °C. All CE separations were performed in a fused-silica capillary (Composite Metal Services, UK), 50 µm I.D., 363 µm O.D., 31.7 cm total length, 17.0 cm to C^{4}D. This is the minimum capillary length for HP^{3D}CE systems. The inner surface of the capillary was covered using INST coating solution (Biotaq, U.S.A.) to prevent electro-osmotic flow (EOF). All the separations were carried out in the optimized background electrolyte (BGE) of 2.0 M acetic acid (pH 2.15). Samples were introduced into the capillary hydrodynamically by the pressure 50 mbar for 3 s. The maximum voltage +30 kV was use for the separation. The sample preparation is based on the ten times dilution of hydrolysed mitochondria by mixture acetonitrile/water (3:1, v/v). Mitochondria were isolated from skeletal muscle of laboratory rats.

3 Results and Discussion

Metformin and phenformin are weak organic bases that migrate as cations in acidic background electrolytes [1]. Consequently, solutions of acetic acid were tested for the CE separation of metformin and phenformin. In addition, C^{4}D detectors exhibit low noise levels and high baseline stabilities in HAc-based BGEs. The BGE composition was optimized directly in separation of mitochondria samples. Of the tested concentration range, 0.1 to 4.0 M HAc, the best results were obtained in 2.0 M HAc. This BGE is also appropriate for determination of propyl triphenylphosphonium bromide (TTP). Large molecules of TTP are often used in mitochondria research because entrance into the mitochondria matrix and uncoupled the Krebs cycle and respiratory chain. The full separation of all compounds of interest together with internal standard, 4-aminopyridine, was completed in the time shorter than 2 minutes.

The sensitivity of CE/C^4D is characterized by limit of detection at submicromolar level for all analytes.

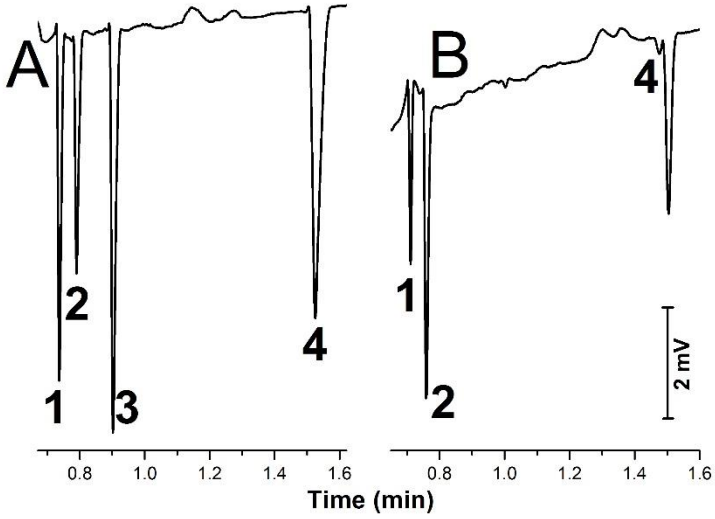

Fig. 2. CE/C^4D analysis of the mixture of standards at concentration 100 μM (A); analysis of mitochondria sample treated by metformin and propyl triphenylphosphonium bromide. Peak identification: metformin (1); 4-aminopyridine (internal standard, 2); phenformin (3) and propyl triphenylphosphonium bromide (4).

4 Conclusions

Electrophoretic separation performed on the short separation path was combined with contactless conductivity detection. This analytical approach is sufficient for the determination of antidiabetic drugs metformin and phenformin together with an uncoupler propyl triphenylphosphonium bromide in mitochondria samples isolated from skeletal muscles of rats.

Acknowledgement

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P98 SEPARATION OF TRYPTIC DIGEST OF CYTOCHROME C WITHIN A LONG NANOELECTROSPRAY TIP

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Summary
Capillary electrophoresis coupled to mass spectrometry (CE-MS) posses powerful tool for separation and detection of wide range of ionic species. To avoid complicated constructions of interfaces we have conducted the separation within a long and thin nanoelectrospray tip [1]. We have investigated main properties of such an experimental design on a complex sample of tryptic digest of cytochrome c.

1 Introduction

This work is aimed at optimization of electrophoretic separation within a long nanoelectrospray tip with mass spectrometric detection. The potential drop needed for sufficient resolution was reached by using system with high resistance.

2 Experimental

The electrophoretic separation was performed in 100 cm x 10 μm I.D. silica fused capillary ended by sharp polished ESI tip. The ESI tip was positioned approximately 2 mm in front of the mass spectrometer sampling orifice (LTQ Velos Pro, Thermo Fisher).

The sample of tryptic digest of cytochrome c from equine heart (Sigma Aldrich, ≥95%) was prepared by common procedure. To make solution free of ammonium bicarbonate used as a buffer it was evaporated at 37°C and dissolved again in 0.01 % formic acid. Sample of concentration 0.12 mg/mL was loaded from a gas pressurized chamber used also to assist the liquid flow inside the capillary during the experiments with nanoelectrospray. As a background electrolyte 0.01% HCOOH was used.

3 Results and Discussion

At our simple experimental design of interfacing a voltage is applied only at the beginning of the capillary and there is no direct control over the voltage at capillary end, which is ended by a nanoelectrospray tip [2].

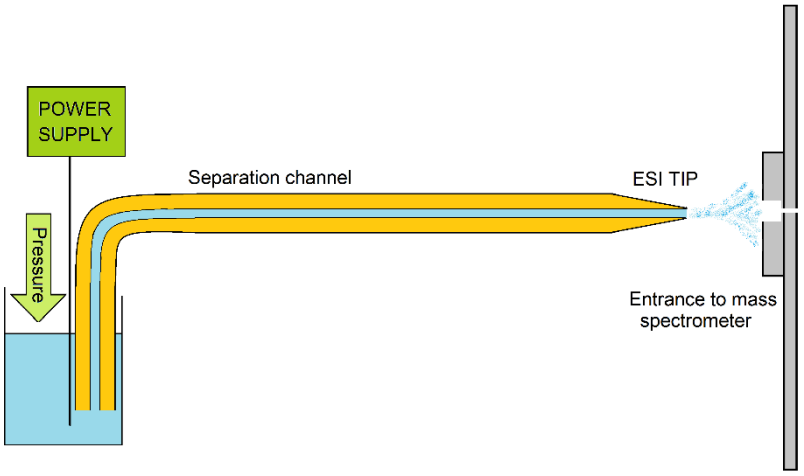

Fig.1. Scheme of used interfacing.

If worked with this type of interface, it is the most challenging to adjust sufficient potential drop within a separation channel and reach optimal voltage on the nanoelectrospray tip at the same time. To meet such conditions it is crucial to work with system of high resistance.

Conductivity of background electrolyte is one of the most important factor influencing resistance. We investigated wide range of potential background electrolytes and eventually 0.01% HCOOH (σ = 265 μS/cm, pH = 3.20) has been chosen as the most appropriate. Not only that it has low conductivity but it still has acidic pH which is desirable for charging of analytes.

For further increase of resistance capillary of I.D. 10 um was used. At the end of the capillary sharp tip was fabricated. There is several ways for fabrication of nanoelectrospray tip (e.g. pulling, etching and grinding). The most promising results were obtained for grinded tips. However, thin capillary brings more than high resistance. Other upsides are ability to create fine electrospray plume, low theoretical plate is reached and it posses more robust system for electrospraying [3].

Velocity of ions during the separation is given by contribution of hydrodynamic flow rate and electrophoretic mobility. To reduce the first named contribution the separation was run for 12 minutes at voltage of 30 kV with no flow rate. Later the conditions were changed to stable plume could be formed. Voltage was decreased to 5 kV and flow rate of 5 nL/min was developed (Fig. 2).

At such experimental conditions we have reached promising resolution and sensitivity. What is more, loaded amount of sample was only 0.93 fmol, which predestine this type of interfacing for analysis of small amount of sample.

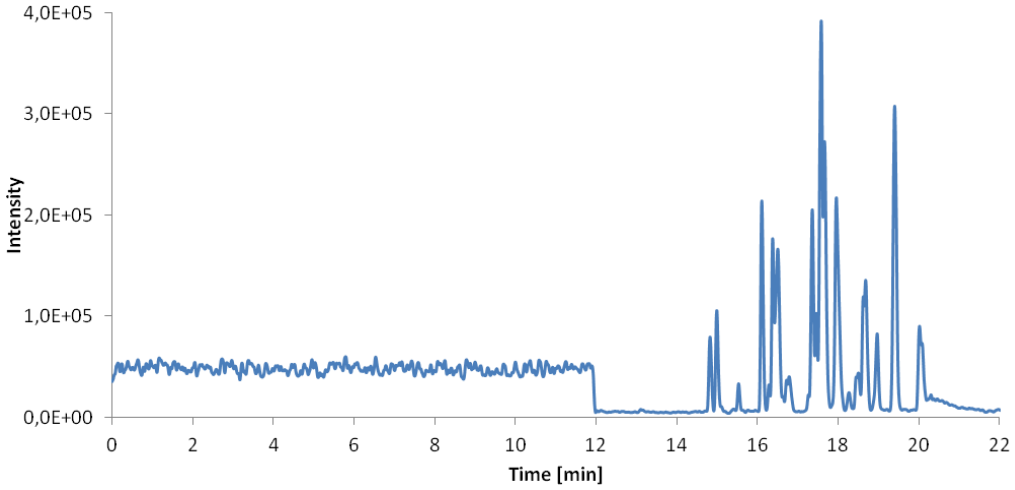

Fig. 2. Base peak electropherogram of tryptic digest of cytochrome c conducted in 100 cm x 10 μm I.D. capillary. Gradient voltage in two steps was applied - 30 kV (12 min) and 5 kV (12-22 min). Flow rate 5 nL/min was developed only during 5 kV step.

4 Conclusions

On a model sample of tryptic digest of cytochrome c was shown that this type of interfacing is viable. In comparison to other approaches it brings construction simplicity and potential to work with small amount of sample.

Acknowledgement

The work has been supported by GACR P20612G014 and CZ.1.07/2.3.00/20.0182.

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P99 SEPARATION OF ASPARTIC A GLUTAMIC ACIDS ENANTIOMERS BY MICROCHIP AND CAPILLARY ELECTROPHORESIS

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Summary
A new microchip electrophoresis (MCE) method for separations of underivatized aspartic (Asp) and glutamic acids (Glu) on a microchip with implemented contact conductivity detection was developed and capillary electrophoresis (CE) with indirect UV detection was used for a comparative study. Achiral separation conditions were optimized in order to eliminate electroosmotic flow (EOF) and to achieve comparable driving force using the same composition of background electrolyte (BGE), i.e. the same carrier and counter ion in both MCE and CE approaches. Chiral methods were developed by adding chiral selector (vancomycin) to the BGE. The conventional CE enabled a complete resolution of Asp and Glu enantiomers, while MCE enabled a partial resolution of D- and L-Asp and a complete resolution of Glu enantiomers on significantly shorter separation path.

1 Introduction

Asp and Glu represent low-abundance chiral compounds of biological significance and can be considered as potential markers of some neurological diseases (Alzheimer`s and Parkinson`s disease [1, 2], kidney dysfunction [3], growth state [4], etc.). In this respect, evaluation of enantiomeric ratio is necessary for reliable predictions of such diseases.

Several CE analytical methods have been developed and optimized within the context of achiral separations of amino acids (AAs) essential for humans [5, 6]. Although, there were same separations performed with unlabeled AAs [7], the most of the papers are dealing with chiral separations of AAs that were performed after their previous derivatization [8, 9].

This work deals with a development of a new microanalytical method for chiral resolution of Asp and Glu on a microchip with conductivity detection and a comparison of developed MCE method with conventional CE with indirect UV detection.

2 Experimental

MCE separations were performed on a poly(methylmethacrylate) (PMMA) channel-coupling microchip (IonChipTM 3.0, Merck, Darmstadt, Germany) with integrated contact conductivity detection. Separations were realized in a hydrodynamically

closed separation system and suppressed EOF by adding methylhydroxyethylcellulose (MHEC) into the BGE with pH 5.2. Separations on a microchip were carried out in the separation channels with inner cross-section of 200-500 × 140-200 μm and total length of 11.5 cm.

CE separations were performed in 75 μm i.d. fused-silica capillaries with commercially derivatized inner surface in a hydrodynamically open system with suppressed EOF. Achiral separations were carried out in the capillary with effective length of 36 cm and chiral separations in the capillary with effective length of 76.5 cm. Detection was performed by on-column UV detector working at 254 nm wavelength. Underivatized Asp and Glu has no chromophore group and therefore BGE used in CE separations was enriched by addition of sorbic acid as absorbing co-ion. Chiral environment was achieved by addition of chiral selector into the BGE optimized for achiral CE and MCE separations.

3 Results and Discussion

Macrocyclic antibiotics (vancomycin, teicoplanin, kanamycin), native and derivatized cyclodextrins (α-, β-, γ-cyclodextrin, sulfated and hydroxypropyl-β-CD) were used in the chiral selectivity studies performed on the microchip. From all of the studied chiral selectors (added to the BGE) only vancomycin provided sufficient chiral selectivity.

Addition of 5 mM of vancomycin to the BGE enabled a baseline resolution of Glu enantiomers (Fig. 1a). An increase of vancomycin concentration to 7.5 mM enabled partial resolution of D- and L-Asp (Fig 1b) on the microchip, as well. CE separations in the capillary with effective length of 50 cm and 5 mM addition of vancomycin into the BGE achieved only resolution of Glu enantiomers. An increase of the length of the capillary up to 76.5 cm enabled to resolve D- and L-Asp (Fig. 1c). Baseline resolution of D- and L-Asp, and D- and L-Glu was achieved with 7.5 mM addition of vancomycin to the BGE (Fig. 1d).

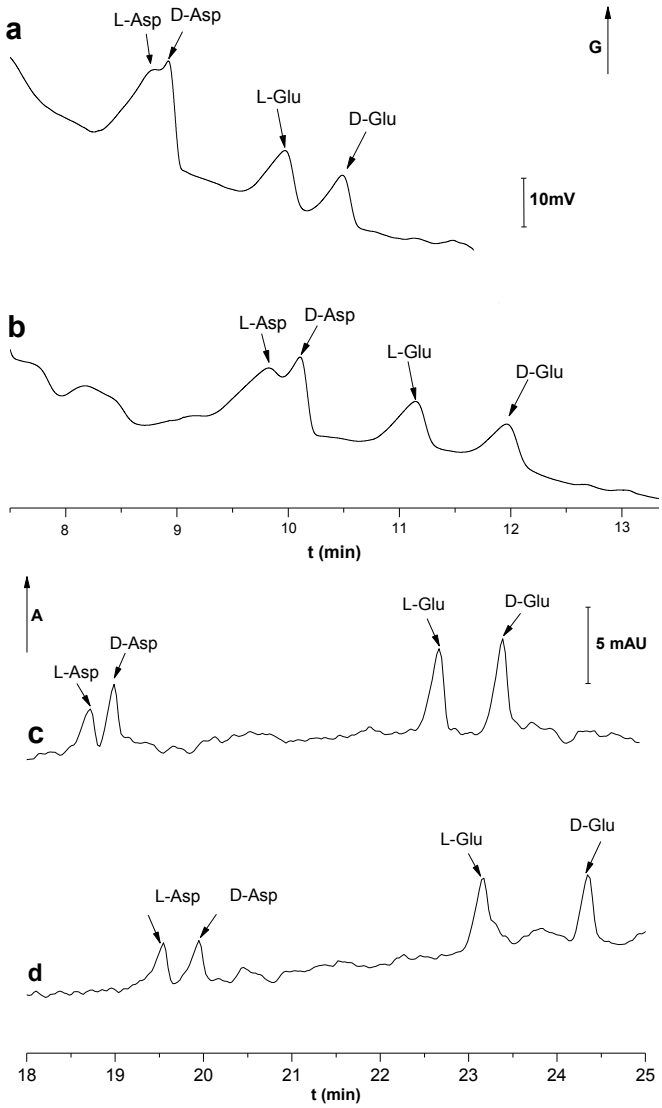

Fig. 1. Chiral MCE and CE separations of standard mixtures of D, L-Asp and D, L-Glu on the PMMA microchip (a, b) and in the capillary (c, d). a) current 35 µA (approx. voltage 5 kV), pH of BGE 5.3, 5 mM vancomycin; b) current 40 µA (approx. voltage 5 kV), pH of BGE 5.3, 7.5 mM vancomycin; c) current 10 µA, (voltage -25 kV), pH of BGE 5.1, 5 mM vancomycin; d) current 8 µA, (voltage -25 kV), pH of BGE 5.1, 7.5 mM vancomycin. Injected sample: 50 µM L-Asp, 50 µM D-Asp, 50 µM L-Glu, 50 µM D-Glu.

4 Conclusions

The conventional CE using the BGE with 7.5 mM vancomycin enabled to achieve a complete resolution of Asp and Glu enantiomers. The same concentration of vancomycin in the BGE used in MCE enabled a partial resolution of D- and L-Asp and a complete resolution of Glu enantiomers. According to the results, MCE performed on the PMMA microchip with universal contact conductivity detection can be considered as a very promising alternative to the conventional CE with indirect UV detection for chiral separations of Asp and Glu enantiomers.

Acknowledgement
This work was supported by grants from the Slovak Research and Development Agency (APVV-0259-12) and the Slovak Grant Agency for Science (VEGA 1/1149/12). This contribution is the result of the project implementation (ITMS 26240220061) supported by the OPRaD funded by the ERDF.

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P100 ANALYSIS OF INDIVIDUAL ORGANELLES

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Summary
Heterogeneity of organelles particularly alterations in their size, quantity or membrane protein expression can be studied using capillary electrophoresis (CE) with laser induced fluorescence detection (LIF). Here we present a method for sample preparation including cell disruption, organelle isolation and antibody labeling for high sensitivity fluorescence detection of individual lysosomes.

1 Introduction

Various types of diseases including cardiovascular and ageing-related diseases, neurodegenerative disorders and cancer are accompanied by defective mechanisms of cellular homeostasis maintenance. Organelles are affected as well during the disease as they are main cellular functional units. Therefore the organelle analysis may become an important tool for investigation of diseases on sub-cellular level.

Analysis of organelles using precisely calibrated CE-LIF instrumentation can provide valuable information, e.g. the average quantity of particular organelle type in one cell, their electrophoretic mobility, surface charge density, morphology, and mitochondrial membrane potential. Using specific antibody labeling and fine-tuned instrument we can monitor the expression of membrane proteins [1].

Lysosomes are involved in conservation of cellular integrity because they are directly responsible for degradation and recycling of cellular waste. Their function stretches even further as they participate in processes like plasma membrane repair or energy metabolism [2].

The aim of this work is to develop a suitable method for lysosome preparation followed by CE-LIF analysis.

2 Experimental

2.1 Cell cultivation

Mouse myoblasts (cell line C2C12) were cultured in Gibco® DMEM Phenol Red medium (Life Technologies, USA) containing10% of fetal bovine serum at 37° C and 5% CO_2.Cells were split every 2-3 days.

2.2 Cell disruption and organelle isolation

Cells were lysed using electrical disruption. After centrifugation and washing steps were cells resuspended in the isolation buffer and pipetted into an electroporation cuvette. To disrupt cell membrane we delivered 60 square wave pulses at 350 $V.cm^{-1}$, pulse duration was 2.5 ms with 1 s gap between pulses. Buffer contained 6 $\mu g.mL^{-1}$ of digitonin to weaken cell membrane by selectively dissolving sterols.

Lysate was then centrifuged at 600 x g for 5 min to spin out large debris. Supernatant containing organelles was collected and centrifuged at 14000 x g for 20 min. The organelle pellet was resuspended in CE buffer.

2.3 Antibody labeling

Organelle suspension was incubated in cold room for 1 h with LAMP2-FITC antibody conjugate (Rat monoclonal antibody to the Mouse CD107b antigen, Molecular Probes, USA). Specific antibody to CD107b antigen (lysosomal-associated membrane protein 2, LAMP2) is conjugated with fluorescein isothiocyanate (FITC). Final concentration of antibody was 5 $\mu g.mL^{-1}$.

2.4 Dual-labeling and two channel detection

This method offers higher level of certainty that correct type of organelle has been labeled. Fluorescent beads or long-chain dextran conjugates can be engulfed by

myoblasts to enter their endocytic pathway. After organelle isolation and antibody labeling the fluorescence emitted by endocytosed beads was detected by first photomultiplier tube (PMT) while the second PMT detected fluorescence of the antibody conjugate.

3 Results and Discussion

The preliminary tests conducted in pressure driven flow-through mode showed that the antibody conjugate is suitable for organelle CE-LIF analysis (Fig. 1) as it provides a detectable signal of labeled lysosomes.

The next valuable observation is that mouse myoblasts can be easily lysed by electrical disruption while leaving the lysosomes preserved for following steps of analysis.

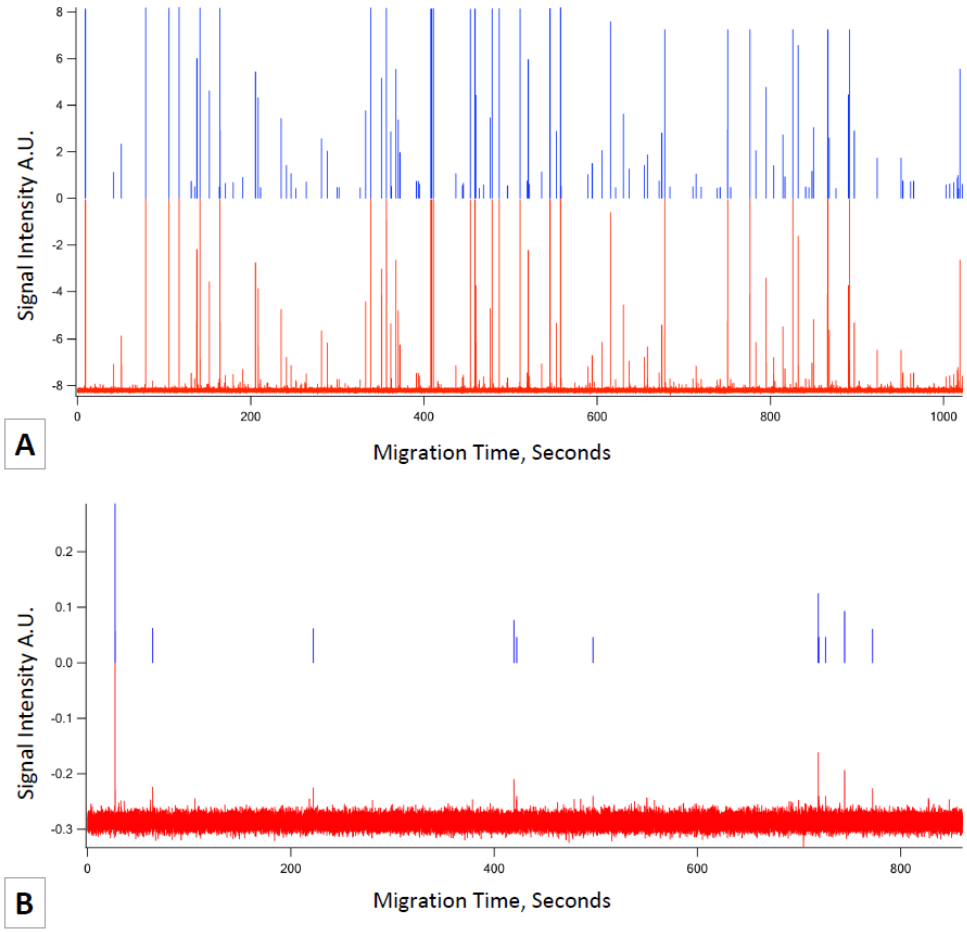

Fig. 1. Pressure driven flow-through experiment. A) Peaks of LAMP2-FITC antibody stained lysosomes, B) Peaks of control, non-stained organelle suspension. PMT voltage: 1000 V, N2 pressure: 10 kPa, protein concentration: 20 µg.mL-1, antibody concentration: 5 µg.mL-1, 50 cm capillary. Red is original curve, blue are positively identified peaks after running 5 σ function.

Fluorescent beads (0.1 μm in diameter) were used for dual-labeling of lysosomes, because mouse myoblasts are able of their endocytosis (Fig. 2).

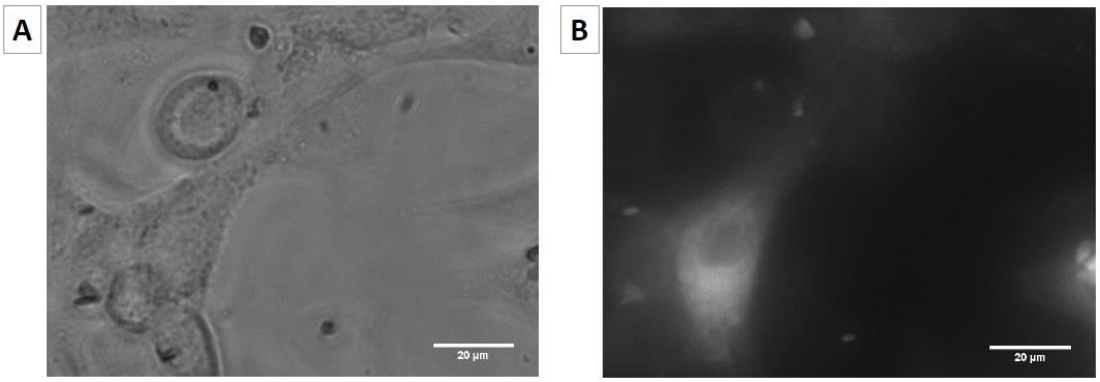

Fig. 2. Confocal microscope images of fluorescent beads endocytosed inside myoblast cells. A) Cells treated with fluorescence beads (BF image, 40x objective, exposure time: 0.2s), B) Cells treated with fluorescence beads (DOX filter, 40x objective, exposure time: 0.1s).

4 Conclusions

This concept of dual-labeling of lysosomes showed first promising results for two-channel simultaneous detection of different organelle types, e.g. mitochondria, autophagosomes. However, it needs to be further optimized; especially the fluorophores has to be selected with emission maxima more distant from each other.

Acknowledgement

Financial support from the Grant Agency of the Czech Republic (14-06319S), the Academy of Sciences of the Czech Republic (M200311201) and the institutional research plan (RVO:68081715) is acknowledged.

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P101 COMPARISON OF DIAGNOSTIC PERFORMACE OF HIGH RESOLUTIN MASS SPECTROMETRY AND TANDEM MASS SPECTROMETRY – A CASE OF PURINE AND PYRIMIDNE METABOLICK DISORDERS

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1 Introduction

Inherited metabolic disorders of purine and pyrimidine (PP) metabolism exhibit broad neurological, hematological, immunological and renal manifestations. They are characterized by increased concentrations of PP metabolites in body fluids caused by abnormal or missing activity of particular enzyme which is involved in their metabolism. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is method of choice in the last 15 years for diagnoses of PP metabolic disorders [1-3]. Due to instrumental price drop high-resolution mass spectrometry (HRMS) is starting to be increasingly used in clinical laboratories. The aim of this work was to compare diagnostic efficacy of these two approaches.

2 Experimental

We tested samples from patients suffering from PP disorders to evaluate performance of the systems. Samples were analyzed on QTRAP 5500 triple quadrupole instrument (AB Sciex, Foster City, CA, USA) and Orbitrap Elite (Thermo Fisher Scientific, San José, CA, USA). Separation was accomplished by HPLC system Dionex UltiMate 3000 (Germering, Germany) using a Phenomenex Kinetex C18, 2.6 µm 2.1 x 150 mm column housed in the oven maintained at 55°C. The chromatographic run was performed at 250 µl/min with a gradient profile of mobile phase consisting of H_2O containing 25 mmol/l acetic acid (eluent A) and an aqueous mixture of 50 % methanol containing 12.5 mmol/l acetic acid (eluent B). Run time of the analysis was 12 min.

3 Results and Discussion

Sensitivity of LC-MS/MS system was in the range of 0 - 3 and for the orbitrap 0 - 2, respectively. Selectivity (measured as number of detected peaks at given optimal settings for both instruments) was 0 - 2 times better for individual diagnostic biomarkers for HRMS compared to LC-MS/MS.

4 Conclusions

HRMS and LC-MS/MS are both useful tools for diagnoses of PP diseases. LC-MS/MS provides more sensitive analyses and HRMS serves better selectivity.

Acknowledgement

This work was supported by grant LF UP 2014-011, grant of IGA Ministry of Health, Czech Republic NT12218. The infrastructural part of this project (Institute of Molecular and Translational Medicine) was supported from NPU I (LO1304).

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P102 THE IMPACT OF FARMING PRODUCTION SYSTEM ON LACTIC ACID BACTERIA AND THE AMOUNT OF ORGANIC ACIDS IN GRAPE MUST DURING FERMENTATION

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Summary

The study is based on determining the impact of farming production system on the number of lactic acid bacteria in grape must during alcohol fermentation by cultivation. Simultaneously organic acids, which significantly affect the organoleptic profile of wine and their quantity is affected by lactic acid bacteria, were determined. Organic acids were analyzed by ion exclusion HPLC.

1 Introduction

Winemaking can be summarized as the biotransformation of must into wine, which is carried out principally by *Saccharomyces cerevisiae* strains during the primary or alcoholic fermentation. A secondary fermentation, the so-called malolactic fermentation (MLF), is biodeacidification that is also often encouraged because it improves wine stability and quality. Malolactic fermentation usually takes place after alcoholic fermentation, but may also occurs simultaneously with the primary fermentation. During the fermentation lactic acid bacteria metabolize malic acid to form lactic acid and CO_2 [1].

Agriculture in Europe dynamic development in the last decades. Production systems are moving towards organic farming systems, which aim to sustain

agricultural production and relationships in ecosystem. It is known that the main factors influencing the occurrence of lactic acid bacteria in grape must are pH, temperature and ethanol content. Given the current trend to switch from conventional to organic farming is good to know if lactic acid bacteria are affected by agrotechnical production system [2, 3].

2 Experimental

The aim of this work was monitoring of total number of lactic acid bacteria occurring in grape must during wine production and simultaneously determining the amount of lactic acid, malic acid and tartaric acid – organic acids, which are particularly affected by lactic acid bacteria in grape must. The study was performed in variety Pinot Noir from both organic and integrated vineyards from the same village and also winemaker to ensure the same environmental conditions.

The determination of the total number of lactic acid bacteria was performed by cultivation on Tomato juice agar and colony forming units determination. Analysis of organic acids in standards and samples was performed by ion exclusion HPLC, where separation is achieved through a combination of ion exclusion and partitioning processes using dilute acidic mobile phases (0,65mM sulfuric acid). The eluting compounds were detected by a UV detector at 210 nm.

3 Results and Discussion

On the first day of fermentation the number of lactic acid bacteria (LAB) was $(52{,}10 \pm 0{,}08) \cdot 10^5$ CFU/ml on the grape wine variety Pinot Noir from organic vineyard and $(9{,}78 \pm 0{,}28) \cdot 10^5$ CFU/ml on the grape wine variety Pinot Noir from integrated vineyard. At the beginning of fermentation the number of LAB decreased in variety from integrated vineyard. In variety from organic vineyard the number of LAB was on third day higher than first day but decreased later. In the final stage of alcoholic fermentation, in the grape wine variety from organic vineyard the number of LAB ranged from $(2{,}03 \pm 0{,}25) \cdot 10^5$ to $(38{,}31 \pm 1{,}00) \cdot 10^5$ CFU/ml and in the grape wine variety from integrated vineyard from $(1{,}77 \pm 1{,}58) \cdot 10^5$ to $(4{,}23 \pm 1{,}03) \cdot 10^5$ CFU/ml (Fig. 1, Fig. 2).

The amount of malic acid in the varieties from different agriculture production did not vary substantially (Fig. 3). However, differences in the amount of lactic acid and tartaric acid were significant (Fig. 4, Fig. 5).

Many authors examined the effect of organic agriculture on biodiversity, the occurrence and activity of microorganisms. Studies of wine were focused mainly on yeasts, fungi and Gram-negative bacteria. The study by Schmidt et al. [4] showed that plant protection in conventional and organic viticulture had an effect on the microorganisms occurring on the grapes. The study aimed to fungi and Gram-negative bacteria showed influence not only on the species composition of microorganisms, but also their activity. Although the impact of the regulated agriculture system on lactic acid bacteria on grapes, grape must and wine has not been elucidated yet. However studies of Ruediger et al. [5] revealed that some pesticides affect the metabolic activity of species *Oenococcus oeni*. Study of Vrček et al. [6] dealing with the

influence of organic and conventional system of viticulture on the content of phenolic compounds concluded that the production system had an impact on the amount of these substances. Phenolic compounds were examined for their potential to control the growth of lactic acid bacteria in wine. This study is another reason to assume that lactic acid bacteria are affected by the applied agrotechnical production system. It has been found that, phenolic compounds can act as an activator or inhibitor of bacterial growth and metabolism depending on their structure.

Experimental results indicate that a production system influence the number of lactic acid bacteria. The metabolic activity of lactic acid bacteria was probably also influenced due to differences in the amount of organic acids.

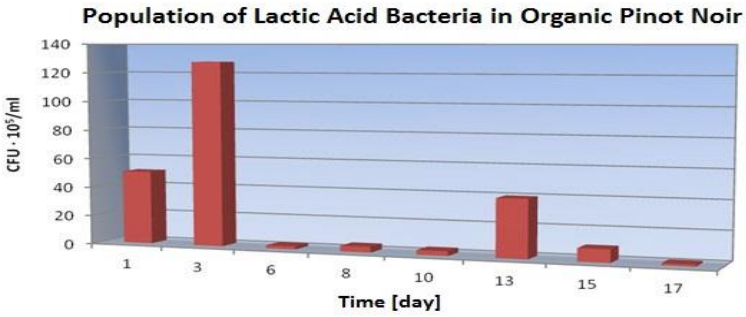

Fig. 1. The number of lactic acid bacteria in Organic Pinot Noir during alcoholic fermentation.

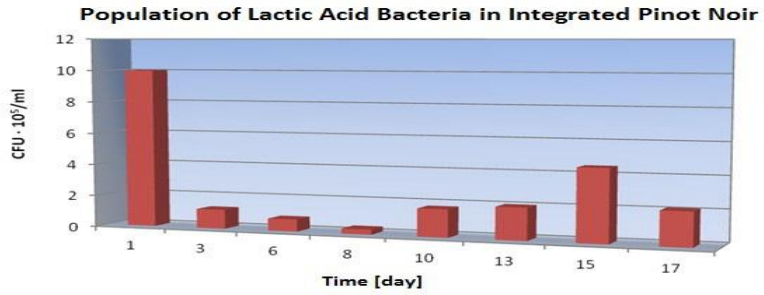

Fig. 2. The number of lactic acid bacteria in Integrated Pinot Noir during alcoholic fermentation.

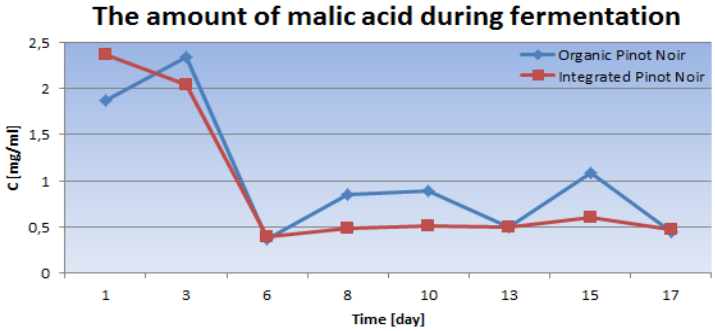

Fig. 3. The amount of malic acid during alcoholic fermentation of variety Pinot Noir from organic and integrated vineyards.

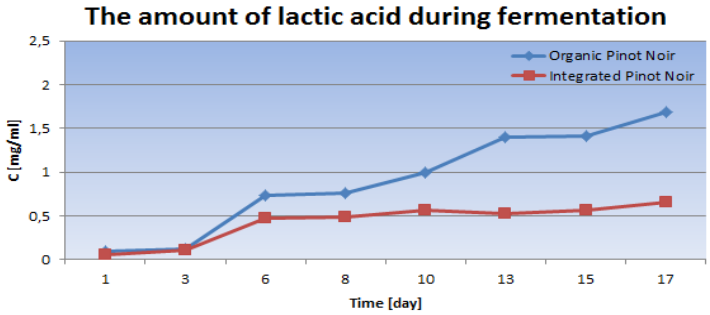

Fig. 4. The amount of lactic acid during alcoholic fermentation of variety Pinot Noir from organic and integrated vineyards.

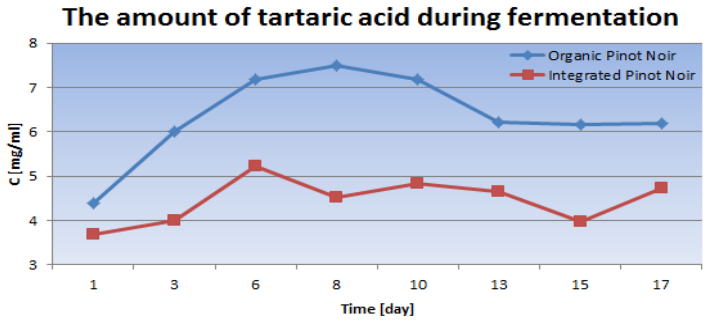

Fig. 5. The amount of tartaric acid during alcoholic fermentation of variety Pinot Noir from organic and integrated vineyards.

4 Conclusions

The experimental results show that the agricultural production system had an impact on the number of viable cells of lactic acid bacteria in grape must during alcoholic fermentation. Differences in the amounts of lactic and tartaric acid during fermentation probably related to number of viable cells of bacteria and their metabolic activity and is closely related to the production system. It could be said that the production system affects the lactic acid bacteria and hence the organoleptic profile of the resulting wine.

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P103 NOVEL UHPLC/MS/MS METHOD FOR THE SAFETY CONTROL OF THE LUPIN SEEDS

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Summary
The presented study reports a novel ultra – high performance liquid chromatography coupled to a triple quadrupole tandem mass spectrometry (UHPLC/MS/MS) method for the quantitation of the quinolizidine alkaloids (namely Lupanine, Lupinine and Sparteine) in different varieties of the *Lupinus* species.

1 Introduction

Legumes represent the main plant source of the proteins in a human diet. They are generally rich in dietary fibre, carbohydrates, vitamins and have the beneficial ratio of the omega - 3, 6 fatty acids. Among the legume seeds, the lupin is a very valuable plant as it is gluten-free, with low fat and low glycemic index. Due to increasing consumption of lupin seeds, flours and lupin containing food, there is a risk of the undesired exposition to quinolizidine alkaloids that unfortunately have hepatotoxic and neurotoxic effects. For this reason, modern analytical methods for a reliable alkaloid determination are highly required.

2 Experimental

A simple extraction technique with 70% (v/v) methanol in water was applied for the isolation of the analytes from the lupin flour. The UHPLC/MS/MS apparatus was obtained from Thermo Fisher Scientific Inc., Waltham, MA, USA. The separation on a C18 reverse-phase column was achieved using a multistep gradient elution with a mobile phase composed of the mixture of 0.05% (v/v) formic acid in water and methanol. The mass spectrometer was operated in Multiple Reaction Monitoring (MRM) mode with positive electrospray ionization (ESI) interface [1, 2, 3].

3 Results and Discussion

Fig. 1 illustrates the comparison of the detection sensitivity in the Full Scan mode (total and extracted ion current chromatograms) and the MRM mode, where the higher sensitivity and selectivity is achieved. The MS^2 spectra of individual lupin alkaloids, obtained by using the high-flow infusion into the mass spectrometer, are illustrated in the Fig. 2. The mass spectrum of Lupanine and Lupinine (Fig. 2A, B) provided a parent ion and 4 product ions, while the Sparteine (Fig. 2C) afforded only one major product ion.

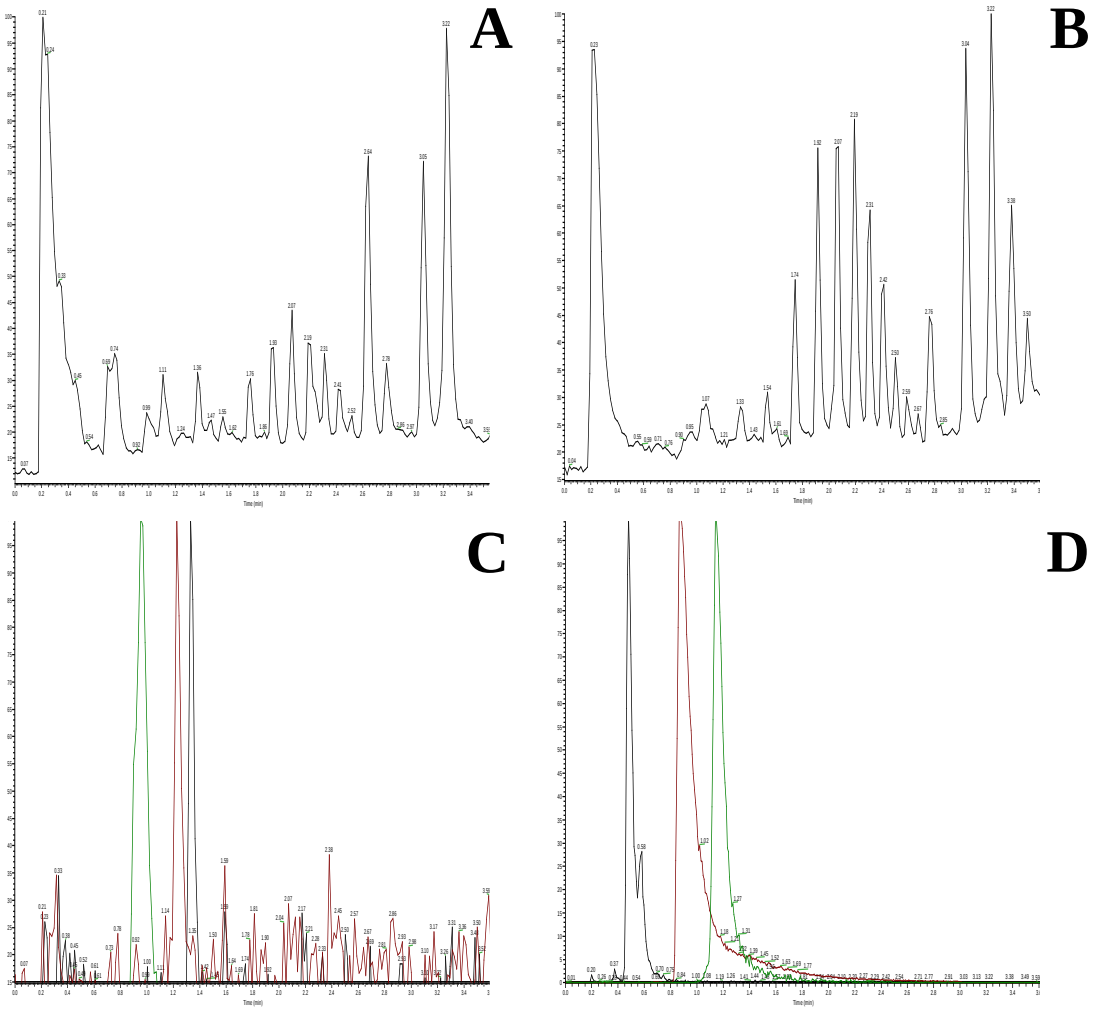

Fig. 1. Positive-ion ESI chromatographic records: A/ total ion current chromatogram (TIC) of water extract; B/ TIC of methanolic extract; C/ extracted ion current chromatogram (EIC) for m/z 170.1 (Lupinine) + 235.2 (Sparteine) + 249.2 (Lupanine); D/ MRM chromatografic records for all 3 lupin alkaloids.

The method was validated for linearity, sensitivity, accuracy, repeatability, robustness and precision. Table 1 summarizes the values of the limit of detection and quantitation (LOD, LOQ), the linear range and correlation coefficients (R^2).

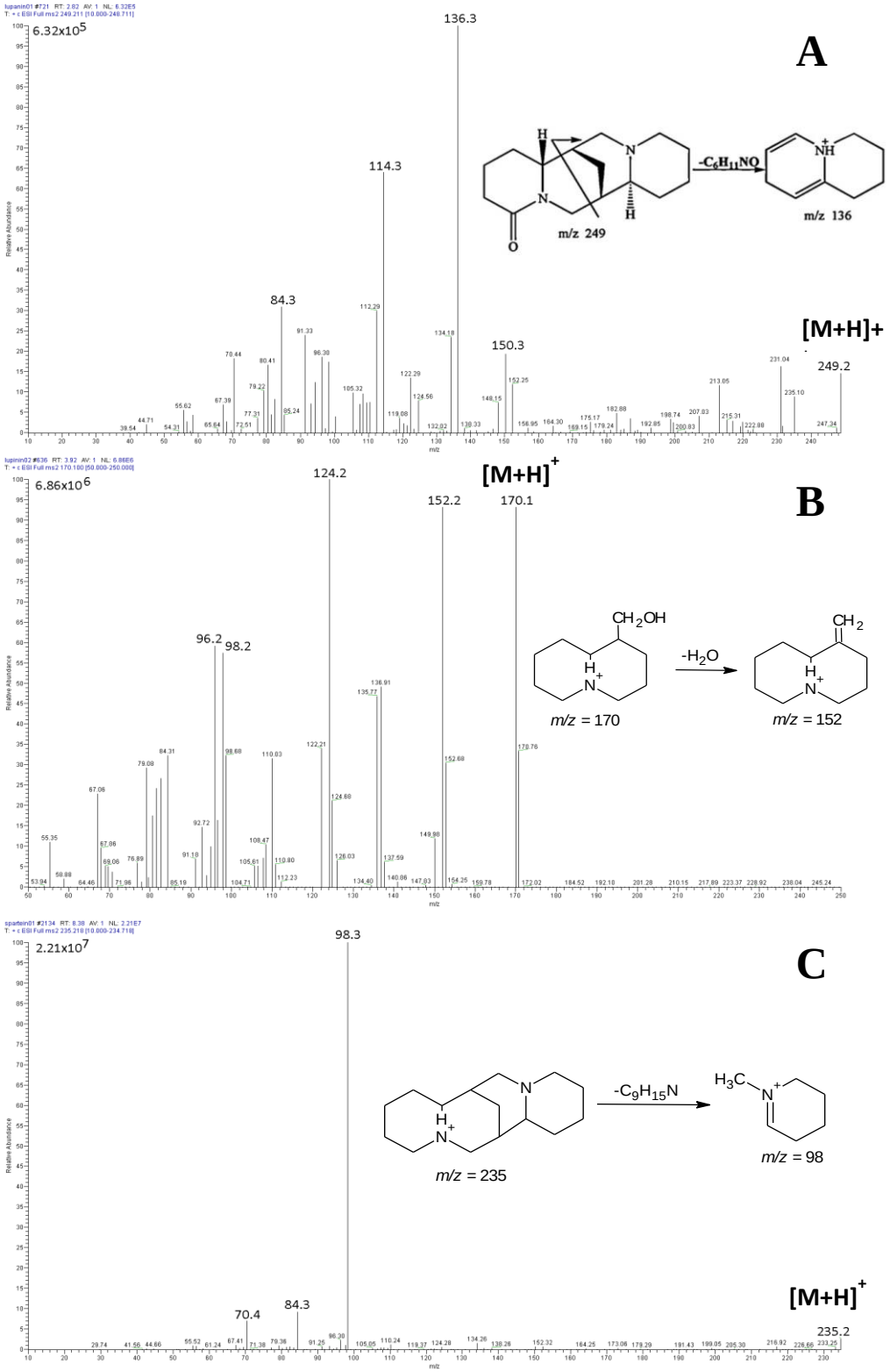

Fig. 2. The MS^2 spectra of individual lupin alkaloids with m/z values of the most important product ions and corresponding fragmentation pathways for: A/ Lupanine, B/ Lupinine, C/ Sparteine.

Table 1. The LOD and LOQ values, the linear range and correlation coefficients (R^2).

Parameter	Lupanine	Lupinine	Sparteine
LOD [μg/ml]	0.0177	0.0219	0.0010
LOQ [μg/ml]	0.0255	0.0326	0.0014
LOD [% w/w]	0.00018	0.00022	0.000010
LOQ [% w/w]	0.00026	0.00033	0.000014
Linear range [μg/ml]	0.0255-1.8144	0.0326-2.836	0.0014-0.044
Correl. Coeff. (R^2)	0.9999	0.9999	0.9977

Our validated method was used to assess the seeds of different *Lupinus* species for their alkaloids content. The amounts of Lupinine and Sparteine were below the LOQ values, while Lupanine was present in all samples within a range from 0.01 to 0.06 % (w/w). The maximum limit of alkaloids content, 0.02 % (w/w), is fixed by the Health Authorities of Australia, New Zealand, Great Britain and France. Three lupin samples exceeded this limit, even though the distributor guaranteed that they are cultivated. This result proves the necessity of the reliable analytical method for the monitoring of the lupin, to prevent possible intake of the neurotoxic alkaloids.

4 Conclusions

The certified UHPLC/MS/MS method for the analysis of the lupin alkaloids was introduced and validated. This methodology provides the appropriate conditions for the safety testing of the lupin seeds used for the preparation of common foods and dietary and nutritional products.

Acknowledgement

This work was supported by Technology Agency of the Czech Republic (grant No. TA01010737).

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P104 RETENTION CHARACTERISTICS OF LABELLED OLIGOSACCHARIDES IN HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY

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Summary
Conditions of hydrophilic interaction liquid chromatopraphy of labelled oligosaccharides (columns, gradient profiles, mobile phase flow and additives) were optimized with the aim of coupling it to the capillary zone electrophoresis in two dimensional system. Different common derivatization reagents used for fluorescent labeling of oligosaccharides including 2-aminobenzamide (2-AB) and 2-aminobenzoic acid (2-AA) we compared. For a separation of labelled oligosaccharides, microcolumns with different stationary phases were tested (Silica gel, Amine, TSK gel amide-80 and GlycanPac stationary phases) Mass spectrometer was used for detection.

1 Introduction

Human body is fulfilled with different types of saccharides (glycans) either in free form or conjugated to proteins or lipids. Conjugated glycans play important role in various biological processes such as cell recongnition, cell-cell interaction, inflammation and disease progression. Glycans can be divided into three basic groups: monosaccharides, oligosaccharides and polysaccharides. Oligosaccharides consist of 2 – 10 monosaccharide units, which are linked by glycosidic bond [1, 2]. Oligosaccharides do not absorb at the wavelength above 200 nm. Therefore it is essential to add chromophore or fluorophore group to a glycan structure for sensitive detection. Oligosaccharides are usually derivatized at their reducing end by reductive amination. Typical derivatization reagents are 2-aminobenzamide (2-AB), 2-aminobenzoic acid (2-AA), 2-aminopyridine (2-PA) and monopotassium 7-amino-1,3-naphthalenedisulfonate hydrate (ANDS) [1, 3]. Such labelled oligosaccharides can be detected either by UV/fluorescent detectors or by mass spectrometer.

2 Experimental

2.1 Apparatus

The separation of oligosaccharides and their derivatives was realized on modular liquid chromatograph which was assembled from degasser, two high pressure pumps, autosampler, mixer and column thermostat (all Shimadzu, Kyoto, Japan). Liquid chromatograph was coupled to a mass spectrometer that was used for detection. Separation of labelled and non-labelled oligosaccharides was realized on four microcolumns with different stationary phases: Ascentis Silica-gel (100 mm x 1 mm,

3µm), TSK gel Amide-80 (100 mm x 1 mm, 5µm), Luna NH_2 (100mm x 1 mm, 3 µm), GlycanPac AXH-1 (150 mm x 1 mm, 1.9 µm).

2.2 Chemicals

Standards of oligosaccharides: maltotriose, isomaltotriose, maltotreaose, maltopentaose, maltohexaose, maltoheptaose were purchased from Sigma-Aldrich (Steinhein, Germany). For a labeling of oligosaccharides, 2-aminobenzoic acid and 2-aminobenzamide, sodium cyanoborohydride, sodium acetate, boric acid and methanol were used (all Sigma-Aldrich). The mobile phase consisted of acetonitrile LC-MS grade (Sigma-Aldrich) and deionized water with various additives, i.e. acetic acid (0.1 %, (v/v)) and ammonium hydroxide (0.1 %, (v/v)) purchased from Penta (Chrudim, Czech Republic).

2.3 Liquid chromatography

Separations of labelled and non-labelled standards of oligosaccharides were tested under both isocratic and gradient conditions in HILIC mode. For isocratic elution, mixtures of water with acetonitrile in concentration range 50% – 80% (v/v) and with addition 0.1% (v/v) actic acid or ammonium hydroxide were used. The mobile phase flow rate in the range between 20 µl/min and 50 µl/min was tested for every microcolumn. In gradient elution, gradient profile was optimized at mobile phase flow 50 µl/min. Columns were thermostated at 35 °C. Injected volume of the sample was 0.5 µl.

2.4 Mass spectrometry

Oligosaccharides and their derivatives were detected (in positive or negative mode) using AB Sciex 4500 QTrap MS with electrospray ionization. Mass spectra were recorded in enhanced MS mode in the range of m/z 400 – 1400 with following conditions: Source temperature: ambient, Curtain gas 20, gas 1 (2): 30 (0), Entrance potential: 10 /-10 V, Scan rate: 1000 Da/s, Declustering potential: 180/-180 V, Collision energy: 30/-30 V.

3 Results and Discussion

The retention of oligosaccharides and their 2-AA or 2-AB derivatives under isocratic conditions on different type of stationary phases was measured and compared in the next step using log k vs log φ (water) plots. An example of such dependence is shown in the Figure 1. Retention characteristics (the slope, m, and the intercept of linear regression, log k_0, representing the hypothetic retention in pure water) of labelled and non-labelled oligosaccharides differ with stationary phases used. Based on comparison of retention of studied oligosaccharides (labelled oligosaccharides) separated on TSK gel Amide-80 and Silicagel microcolumns, it is evident that the retention characteristics (i.e. the m, log k_0 parameters) are much higher in case of TSK gel Amide-80 microcolumn. Separation of oligosaccharides with gradient elution on the column TSK gel Amide-80 is illustrated in the Figure 2.

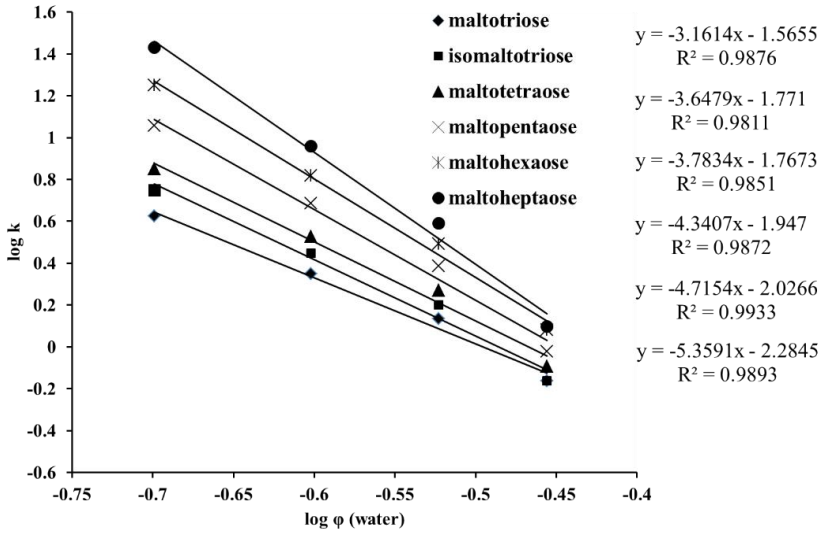

Fig. 1. Dependence between log k and log φ (water). Isocratic separation of oligosaccharadies on TSK gel Amide-80 column. Mobile phase flow 50 µl/min.

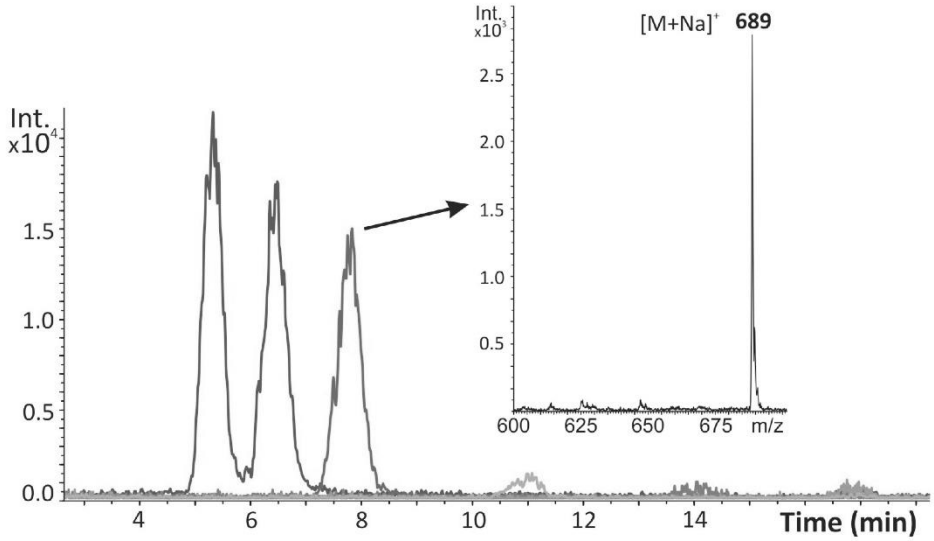

Fig. 2. Seapartion of oligosaccharides (maltotriose, isomaltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose – peaks in this order) on the column TSK gel Amide-80, mobile phase flow 50 µl/min, gradient profile: 0 min 30 % water with 0.1 % acetic acid (v/v)/ acetonitrile – 25 min 50 % water with 0.1 % acetic acid/ acetonitrile.

4 Conclusions

Conditions of HILIC separation of fluorescently labelled oligosaccharides using microcolumns coupled with mass spectrometric detection were evaluated. Among the columns tested, the amide stationary phase in combination with gradient of acetonitrile/water with addition of acetic acid provided the highest resolution of studied oligosaccharides. The obtained retention characteristics served as input data for optimization of 2D system coupling on-line LC with capillary electrophoresis.

Acknowledgement
The work was financially supported by the Czech Science foundation, project No. 14-06319S and project SGFchT06/2013 of University of Pardubice.

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P105 CAPILLARY ELECTROPHORETIC SEPARATION OF FLUORESCENTY LABELLED OLIGOSACCHARIDES

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Summary
Capillary zone electrophoresis was used in this work for the characterization of oligosaccharide mixtures. The aim of this work was to optimize the conditions of separation of oligosaccharides including derivatization step, use of coated or non-coated capillary, length of capillary, composition of background electrolyte and applied voltage.

1 Introduction
The aim of this work was to optimize the conditions of separation of oligosaccharides by capillary zone electrophoresis (CZE). Oligosaccharides play an important role in many cellular events as effective components of the information-carrier molecules in extra-celular-signalling, recognition, trafficking, adhesion and proliferation phenomena [1]. One major limitation of the CZE analysis of oligosaccharides is detection because the oligosaccharides lack of chromophoric activity. To overcome this problem, oligosaccharides are often labelled with UV active and/or fluorescent agents for increased sensitivity [2]. In this work, 2-aminobenzoic acid, 2-amino benzamide, 2-aminopyridine and 7-aminonaphtalen-1,3-disulfonic acid were tested as derivatization agents. Results of the separations were compared and the optimal conditions including derivatization agent, type and length of a capillary, composition of background electrolyte and applied voltage for the separation of oligosaccharide mixtures were selected.

2 Experimental

D-(+)-rafinose, D-(+)-melezitose, maltotriose, isomaltotriose, stachyose (all Dp = 3), maltotetraose (Dp = 4), maltopentaose (Dp = 5), maltohexaose (Dp = 6) and maltoheptaose (Dp = 7) were obtained from Supelco (Bellefonte, PA, USA). 2-Aminobenzoic acid (2-AA), 2-amino benzamide (2-AB), 2-aminopyridine (2-AP), 7-aminonaphtalene-1,3-disulfonic acid (ANDS), ammonium formate, ammonium acetate, acetic acid, ammonium bicarbonate, ammonia, hexadecyltrimethylammonium bromide and sodium cyanoborohydride were purchased from 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) and CTAB were obtained from Janssen Chimica (Beerse, Belgium). Deionized water was prepared using Ultra CLEAR UV apparatus (SG, Hamburg, Germany). The oligosaccharides were derivatized using 2-AA, 2-AB, 2-AP and ANDS agents according to the slightly modified procedure described elsewhere [3, 4, 5].

All experiments were carried out using capillary electrophoresis Agilent ^{3D}CE with UV/Vis diode array detector in non-coated fused silica capillary or polyvinylalcohol and polydimethylacrylamide coated capillaries from Agilent (Palo Alto, CA, USA). Different background electrolytes were used for non-coated or coated capillaries. Phosphate buffer (10, 20 and 50 mmol/L, pH 2.5), ammonium formate buffer (50, 100 and 200 mmol/L, pH 3.0) and ammonium acetate buffer (50, 100 and 200 mmol/L, pH 4.0) were used in combination with non-coated fused silica capillary. Phosphate buffer (10, 20 and 50 mmol/L, pH 7.0), ammonium carbonate buffer (50, 100 and 200 mmol/L, pH 8.0) and 4-methylmorpholine buffer (50, 100 and 200 mmol/L, pH 7.4) were used for separation of oligosaccharides in coated capillaries. The value of pH was measured using Metrohm 827 laboratory pH meter equipped with Unitrode electrode (Metrohm, Herisau, Switzerland). Separation was carried out at 20 °C, applied voltage was -20 kV and -30 kV. The detection wavelength was set to 235 nm.

3 Results and Discussion

The ANDS, 2-AB, 2-BB and 2-AP derivatives of selected oligosaccharides were analyzed using capillary zone electrophoresis. The best results, i.e. efficiency and resolution of derivatives, were obtained with ANDS in phosphate buffer as background electrolyte. In the Fig. 1, there are separations of ANDS derivatives. Fig. 1B shows the separation of oligosaccharide derivatives in non-coated capillary (l_d = 42 cm) with applied voltage -20 kV. The compounds were separated in 15 minutes. Adjustment of the separation conditions (length of capillary l_d = 9 cm, voltage -30 kV) led to shorten time of separation (Fig. 1A).

The dependence of the effective mobility of ANDS derivatives on the concentration of background electrolyte is shown in Fig. 2. Fig. 2A shows behavior of the oligosaccharide derivatives in ammonium acetate buffer (pH 4.0) and Fig. 2B shows the dependence of effective mobility of derivatives in ammonium formate

buffer (pH 3.0). The effective mobility increases with increases concentration of background electrolyte in both cases.

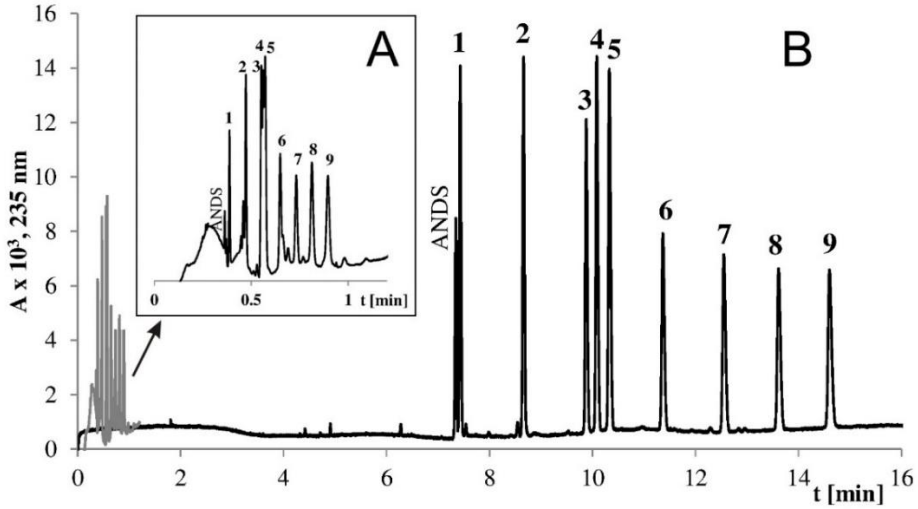

Fig. 1. CZE separation of oligosaccharide derivatives. 1 - D-(+)-Melezitose, 2 - D-(+)-Rafinose, 3 - Maltotriose, 4 - Stachyose, 5 - Isomaltotriose, 6 - Maltotetraose, 7 - Maltopentaose, 8 - Maltohexaose, 9 - Maltoheptaose. A: 50 mmol/L phosphate buffer pH 2.5, non-coated capillary I.D. = 50 µm, lt = 30 cm, ld = 9 cm, voltage -30 kV. B: 50 mmol/L phosphate buffer pH 2.5, non-coated capillary I.D. = 50 µm, lt = 50 cm, ld = 42 cm, voltage -20 kV.

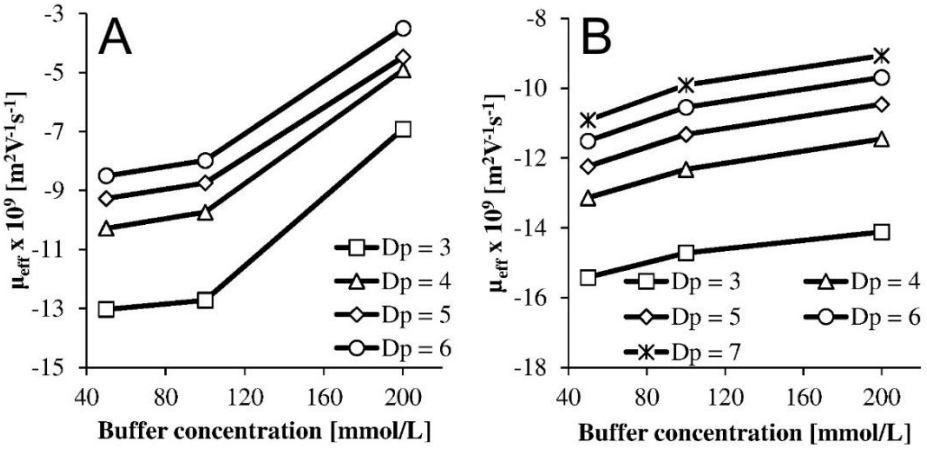

Fig. 2. Dependence of the effective mobility (ANDS derivatives) on the concentration of background electrolyte. A: Ammonium acetate buffer pH 4.0. B: Ammonium formate buffer pH 3.0.

4 Conclusions

Capillary zone electrophoresis was used for the separation of the mixture of oligosaccharide derivatives. 2-Aminobenzoic acid, 2-amino benzamide, 2-aminopyridine and 7-aminonaphtalen-1,3-disulfonic acid were used as derivatization agents. The effects of the type and length of capillary, background

electrolyte and applied voltage on the separation of oligosaccharide derivatives were studied.

The best separation of ANDS derivatives was achieved in phosphate buffer in non-coated capillary. The ANDS derivatives were suitable for fast separation of selected oligosaccharides. The fastest separation in less than 1 minute was achieved in phosphate buffer (pH 2.5).

Acknowledgement
The work was financially supported by the Czech Science Foundation, project No. 14-06319S.

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P106 METABOLOMIC APPROACH TO THE STUDY OF NANOPARTICLES IMPACT TO BARLEY PLANTS

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Summary
Barley plants in the pots after formation of second leaves were exposed in the special chamber to elevated concentration of CdO nanoparticles. After three weeks exposure the content of primary metabolites was measured using metabolomic approach and compared with the control.

1 Introduction

Nanoparticles (NPs) are characterized by their small size (<100 nm) and large surface area, which confer specific physicochemical properties to them. NPs can be derived from natural or anthropogenic sources, such as engineered or unwanted/incidental NPs. The increasing applications and use of NPs are directly related to their release into all compartments of the environment. The toxicity and degradation of these compounds in the environment cannot be accurately assessed yet because it depends on the NPs type, their physicochemical properties, and also on the environmental media in which they partition and the respective conditions.

The effects of NPs have been described in a wide variety of organisms [1-3] however, interactions of NPs with plants have been poorly studied and so the general consequences of NPs exposure for plants remaining unclear. Very few NPs and plant species have been studied and most of the studies reported the effect of nanomaterials on the very early growth stages of the plants, especially on seed germination [4], root elongation and biomass [5]. Majority of papers have focused on phytotoxicity of metal based engineered nanomaterials, but surprisingly negligible interest is given to nanoparticles emitted from technological processes outside to the environment, inclusive of Cd and its oxides, which are abundantly present in ambient particles [6].

Transport of NPs through environment partition is the most critical parameter to evaluate NPs impact and it is expected that NPs which are released into the environment have a high mobility. As compared to algae or fungi, plants might also be exposed to NPs in atmospheric environment but the majority of studies conducted on plants thus far employed an aqueous solution [7, 8], agar [9, 10] or soil [11] rather than air media. Therefore the main goal of our study was to estimate the impact of CdO nanoparticles entering the young barley plants from the air, water and soil. To the best of our knowledge this is the first report on the effect of CdO nanoparticles entering plants from the atmosphere.

2 Experimental

2.1 Preparation of CdO nanoparticles

CdO nanoparticles (CdONPs) were generated continuously in-situ in a hot wall tube flow reactor, using an evaporation–oxidation–condensation technique in which a ceramic crucible containing a small amount of bulk cadmium was placed inside the ceramic work tube of a vertically orientated furnace (Carbolite TZF 15/50/610). The formed cadmium oxide nanoparticles were diluted with a stream of air (20 L/min) and used for whole experiment in dose-concentration chambers. The concentration of CdONPs used in experiment was stable at the concentration level $2.03 \pm 0.45 \times 10^5$ particles/cm^3. A particle size was in the range of 7-60 nm.

2.2 Experimental design of exposure to CdONPs

Barley seeds were germinated, planted into pots and after formation of second leaves the pots were transferred into experimental chamber. In each pot were three barley plants and for each variant there were 10 pots with barley. 30 plants were placed in control cage with no CdONPs and 90 plants were exposed to the same concentration

of CdONPs with following changes: in the first treatment (I_1) CdONPs affected only surface of the plants, in the second treatment (I_2) nanoparticles affected surface and the substrate of the plants and in the third treatment the last 30 plants (I_3) were completely exposed to the effect of CdONPs including plants surface, surface of the substrate and surface of the water into which the pots were immersed. Barley plants were exposed to CdONPs for three weeks, during this time irradiation was 150 μmol m^2 s^{-1} and illumination followed periods 12/12 hours.

After the experiment, leaves and roots were separated and part of the samples was frozen in liquid nitrogen for further metabolomic analysis. Another part of samples was dried and used for determination of Cd content and for electron microscopy study.

2.3 Analysis by liquid chromatography- mass spectrometry

Samples were analysed twice on HPLC-HRMS with positive and negative polarity of MS-Orbitrap. For the high performance liquid chromatography part, a Dionex Ultimate 3000 was used. The column used was a Hypersil Gold column 150mm x 2.1mm, 3μ. MS and MS^n experiments were performed using a LTQ Orbitrap XL-high resolution mass spectrometer equipped with a HESI II (Heated electrospray ionization) source.

3 Results and Discussion

Our established method for the metabolomic studies allows simultaneous analysis of 16 amino acids, 9 polyphenols, 7 acids of Krebs cycle, pentoses, hexoses, disaccharides and 2-deoxy-D-ribose from the group of saccharides, all mentioned compounds except polyphenols are plants primary metabolites.

Most of the results regarding content and composition of primary metabolites revealed large differences on both leaves and roots between control and CdONPs treatment. As an example it can be seen from the Fig. 1 large difference in amino acids composition in the leaves (control vs leaves exposed to different treatments). Two most abundant amino acids are tryptophan and phenylalanine most probably due to their role as precursors for biosynthesis of secondary metabolites. Similar picture was obtained also for the roots of affected plants. In contrary to our working hypothesis the main uptake of CdONPs was in the case of their complete exposure. Detailed study containing all data, including fatty acids profile, Cd content in the leaves and roots and materials from electron microscopy will be published elsewhere.

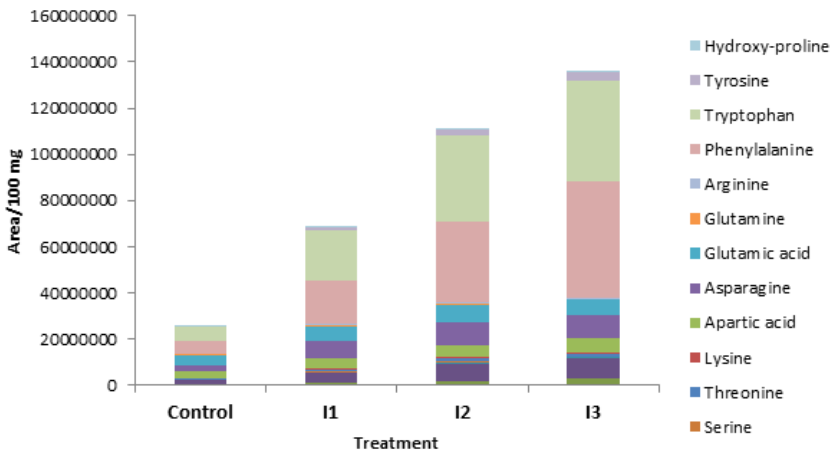

Fig. 1. Amino acids content and composition in the leaves exposed to CdONPs during different treatments (see experimental part). Values are means of 10 replicates.

4 Conclusions

The original hypothesis was that the nanoparticles enter the leaf through the stomata. This is not yet confirmed and it seems that the main uptake of CdONPs was through the roots from the soil and water. The results show differences on both leaves and roots between control and CdONPs treatment. The greatest effect was in the treatment I_3, in which plants were completely exposed to the effect of CdONPs including plants surface, surface of the substrate and surface of the water into which the pots were immersed.

Acknowledgement

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P107 IDENTIFICATION AND QUANTIFICATION OF AROMA COMPOUNDS OF SEA BUCKTHORN BERRIES

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Summary
Thirteen cultivars of sea buckthorn berries were tested for content of volatile aroma compounds using SPME-GC-FID method during two consequent years (2011-2012). In total 76 volatile compounds were identified: 26 alcohols, 13 aldehydes, 14 ketones, 9 acids and 14 esters. Alcohols, ketones and acids were quantitatively the most predominant group of compounds. Significant differences ($p < 0.05$) were found among varieties in both years, Krasavica cultivar was found as having the highest content of aroma compounds, stable during the monitored period.

1 Introduction

Fruits of sea buckthorn (*Hippophae rhamnoides* L.) belong to the most nutritious berry fruits, which seem to have preventive effects against many diseases, e.g. cardiovascular, mucosa and/or skin problems [1]. They are considered to be a good source of large number of bioactive substances like vitamins, carotenoids, phytosterols, organic acids, polyunsaturated fatty acids and some essential amino acids [2]. Besides the nutritional value, the sensory quality of fruits (especially flavour) is very important for consumers. Tang et al. [3] found astringency, sourness and bitterness as the main flavour attributes of sea buckthorn; its aroma was described variously, e.g. as strawberry-, peach-like [3], exotic fruit, pineapple and/or citrus fruit-like [1]. Despite highly acidic and exotic flavour, sea buckthorn berries have good potential for industrial production of various products like juice, tea, syrup, jam, jelly and many other nutritious products [2]. For this reason the pilot growing was started in Czech Republic several years ago.

Flavour of fruits is influenced by volatile aroma compounds present. The literature dealing with the aroma compounds of sea buckthorn is very scarce, firstly studied in the 80s by Hirvi and Honkanen [4]. Then recently several others [5-7] published results about aroma composition of sea buckthorn. The aim of this work was to identify, quantify and compare the volatile aroma compounds of several new cultivars of sea buckthorn berries, especially grown in Czech Republic. Aroma compounds were assessed using GC-FID method connected with SPME extraction.

2 Experimental

2.1 Samples analyzed

The sea buckthorn varieties were grown in breeding institute of Mendel University in Brno (Lednice, CZ). In total 13 cultivars were analyzed (see Fig. 1). The berries were handpicked fully ripe during the seasons 2011-2012, immediately frozen at −15°C and stored until analysis.

2.2 SPME-GC-FID conditions

Volatile compounds were extracted by solid phase microextraction (SPME), identified and quantified using standards by GC-FID. SPME conditions: CAR^{TM}/PDMS fiber 85 μm (Supelco, Bellefonte, Pennsylvania, USA) at 35 °C 20 min, 1 g of the sample, desorption at 250 °C 5 min. GC conditions: gas chromatograph TRACE^{TM} GC (ThermoQuest, Milan, Italy) with a capillary column DB-WAX (30 m × 0.32 mm × 0.5 μm) (J. & W. Scientific, Folsom, CA, USA), injector 250 °C, split-less desorption 5 min, carrier gas N_2, 0.9 mL min^{-1}, flame ionization detector (FID) 220 °C. The oven ramp temperature: 40 °C for 1 min, 5 °C/min up to 200 °C and maintained for 7 min.

3 Results and Discussion

Hirvi and Honkanen [4] firstly identified a total of 60 components in sea buckthorn samples, esters, particularly ethyl and 3-methylbutyl esters, were the most numerous. These compounds display an aroma typical of many fruits and berries. Volatiles of sea buckthorn were also studied by Tiitinen et al. [5]. A total of 45 headspace compounds were identified. The main compounds were esters of branched or straight chain aliphatic alcohols and acids. Similarly Wang et al. [6] found higher alcohols, ethyl esters, acetates, fatty acids, and carbonyl compounds.

In total 76 volatile compounds were identified in this study: 26 alcohols, 13 aldehydes, 14 ketones, 9 acids and 14 esters. In contrast to other authors [5,8], who identified esters as the main compounds, alcohols were the most numerous compounds. As can be seen in Fig. 1, alcohols, ketones and acids were quantitatively the most important group of compounds. Other groups (aldehydes, esters) create less than 1 % of the total content of aroma compounds. Statistical analysis showed significant differences ($p < 0.05$) among cultivars in both years. Krasavica seems to be the cultivar with the richest content of aroma compounds, Hergo and Velkoosecky were found as having the lowest content.

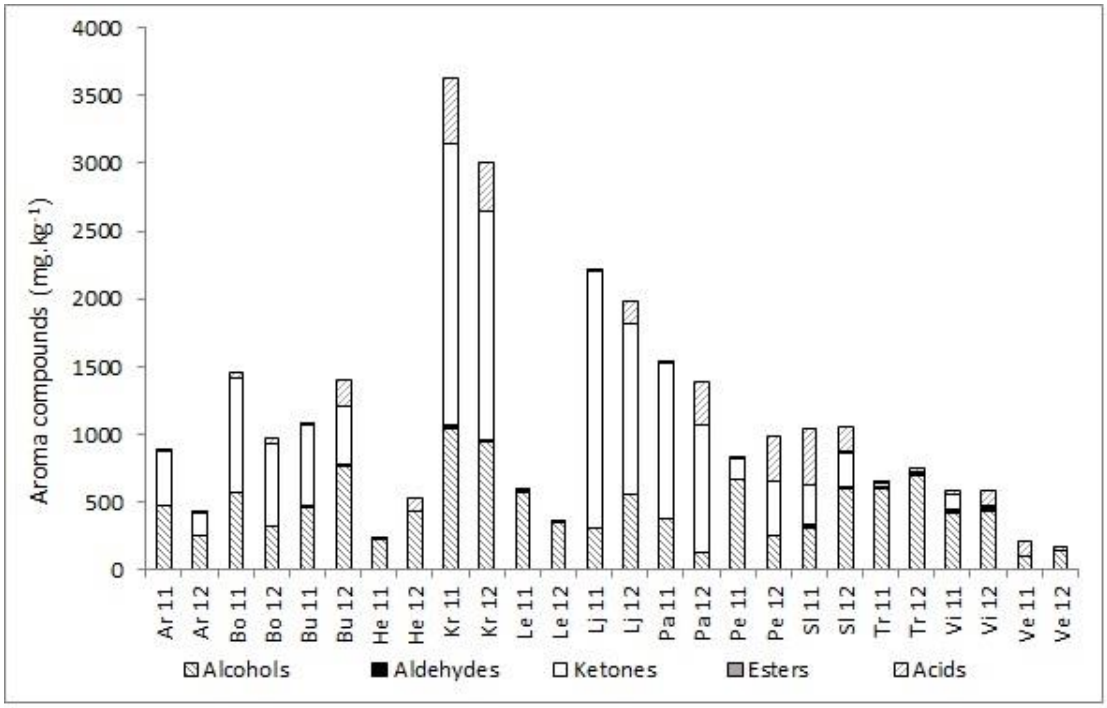

Fig 1. The comparison of groups of compounds identified in sea buckthorn cultivars (picking years 2011-2012). Cultivars: Ar-Aromat, Bo-Botanicky, Bu-Buchlovicky, He-Hergo, Kr-Krasavica, Le-Leicora, Lj-Ljubitelna, Pa-Pavlovsky, Pe-Peterbursky, Sl-Sluničko, Tr-Trofinovsky, Vi-Vitaminnaja, Ve-Velkoosecky.

4 Conclusions

Although several articles about aroma compounds of sea buckthorn berries have been published up to now, this work is focused on selected cultivars intended to grow in Czech Republic. In terms of preliminary evaluated sensory quality and nutritive value (results not included) and taking into consideration the content of aroma compounds identified, Krasavica seems to be the most promising cultivar recommendable for growing on a large scale.

Acknowledgement

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P108 THE STUDY OF POLYMORPHISM OF CASTOR (*RICINUS COMMUNIS* L.) USING RAPD TECHNIQUE

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1 Introduction

Castor (*Ricinus communis* L.) is an important industrial oilseedcrop. Its seed oil has multifarious applications in production of wide industrial products ranging from medicines to lower molecular weight aviation fuels, fuel additives, biopolymers and biodiesel [1] - [2]. Castor oil is the only vegetable oil that contains up to 85% of the unique hydroxy fatty acid, ricinoleic acid, which confers distinctive industrial properties to the oil. Castor grows as an indeterminate annual or perennial depending on climate and soil types in tropical, sub-tropical and warm temperate regions in the world [3]. Random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) markers on the other hand, require only small amounts of DNA sample without involving radioactive labels and are simpler as well as faster [4]. RAPD was proven to be quite efficient in detecting genetic variations and used for diversity assessment and for identifying germplasm in a number of plant species [5]-[6]. ISSR has been shown to provide a powerful, rapid, simple, reproducible and inexpensive means to assess genetic diversity and identify differences between closely related cultivars in many species [7] - [8]. Recently, studies have been initiated on assessment of genetic variation in castor germplasm using AFLP and SSR markers [9].

The aim of this study was to assess genetic diversity within the set of 40 ricin genotypes using 8 RAPD primers.

2 Experimental

2.1 Plant material and DNA extraction

Ricin lines (40) were obtained from the breeding station Zeainvent Trnava Ltd. (Slovakia). DNA of 40 genotypes of castor was extracted from 10 day old leaves using the Gene JET Plant Genomic DNA Purification Mini Kit.

2.2 RAPD amplification

Amplification of RAPD fragments was performed according to [12] (Table 1) using decamer arbitrary primers (Operon technologies Inc, USA; SIGMA-D, USA). Amplifications were performed in a 25 µl reaction volume containing 5 µl DNA (100 ng), 12.5 µl Master Mix (Genei, Bangalore, India), and 1 µl of 10 pmol of primer. Amplification was performed in a programmed thermocycler (Biometra, Germany) with initial denaturation at 94 °C for 5 min, 42 cycles of denaturation at 94 °C for 1 min, primer annealing at 38 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. Amplified products were separated in 1.5% agarose in 1× TBE buffer. The gels were stained with ethidium bromide and documented using gel documentation system Grab-It 1D pre Windows.

2.3 Data analysis

A dendrogram based on hierarchical cluster analysis using the unweighted pair group method with arithmetic average (UPGMA) with the SPSS professional statistics version 17 software package was constructed. For the assessment of the polymorphism between genotypes ricin and usability RAPD markers in their differentiation we used diversity index (DI) [10], the probability of identity (PI) [11] and polymorphic information content (PIC) [12].

3 Results and Discussion

PCR amplifications using 8 RAPD primers produced 66 DNA fragments that could be scored in all genotypes (Fig 1). The selected primers amplified DNA fragments across the 40 genotypes studied, with the number of amplified fragments ranged from 3 (OPE-07) to 13 (SIGMA-D-01), and the amplicon size ranged from 100 to 1200 bp (Table 2). Of the 66 amplified bands, all 66 were polymorphic, with an average of 8,25 polymorphic bands per primer. The polymorphism information content (PIC) value ranged from 0.556 (OPE-07) to 0.895 (OPD-13), with an average of 0,784 and index diversity (DI) value ranged from 0,621 (OPE-07) to 0,896 (OPD-13) with an average of 0,798 (Table 1).

A dendrogram based on hierarchical cluster analysis using UPGMA algorithm separated 40 genotypes into two groups. Cluster I contains four genotypes, in which separated a single ricin genotype RM-53 from other three genotypes (RM-55, RM-61 and RM-84). Cluster II was divided into two subclusters (2a, 2b) and separated one unique genotype RM-76. Subcluster 2a contains three ricin genotypes and subcluster 2b contains 32 genotypes of ricin. We could not distinguish 2 genotypes, RM-64 and RM-75, which can be caused due the same genetic background, which were grouped in 2b subclaster. For better diferentiation of analyzed ricin genotypes, it is necessary to use a higher number of RAPD markers.

Table 1. The statistical characteristics of the RAPD markers used in castor.

RAPD Primers	Number of alleles	DI	PIC	PI
OPA-02	7	0,788	0,776	0,013
OPA-03	9	0,861	0,858	0,010
OPA-13	7	0,818	0,809	0,025
OPD-07	8	0,741	0,740	0,025
OPD-13	12	0,896	0,895	0,001
OPE-07	3	0,621	0,556	0,074
SIGMA-D-01	13	0,886	0,884	0,002
SIGMA-D14	7	0,767	0,749	0,055
Average	**8,25**	**0,798**	**0,784**	**0,027**

4 Conclusions

The analysis showed that the RAPD markers are very effective molecular markers for the assessment of the genetic diversity in castor bean. The dendrogram prepared based on UPGMA algorithm divided 40 analyzed genotypes into two main clusters. Cluster I contain 4 genotypes of ricin and cluster II contain 36 ricin genotypes. Using 8 RAPD markers only two castor bean genotypes have not been distinguished. For better discrimination of the analyzed ricin genotypes, it is necessary to use a higher number of RAPD markers. Our analysis proved utilization of RAPD markers for differentiation of used set of castor genotypes. RAPD markers are useful in the assessment of castor bean diversity, the detection of duplicate sample in genotype collection, and the selection of a core collection to enhance the efficiency of genotype management for use in castor bean breeding and conservation.

Acknowledgement

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P109 ANALYTICAL POTENTIAL OF SEPARATION TECHNIQUES AND MASS SPECTROMETRY IN THE STUDY OF BIOLOGICALLY ACTIVE SUBSTANCES

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1 Introduction

At the present at least 8 strains of murine gammaherpes virus (MuHV-4) exist, but complete sequence and genome analysis is known only for MHV-68 [1]. Recently, some monoclonal antibodies (moabs) directed against glycoprotein B (gB) of MHV-68 have been characterized. Some of them, especially those recognizing N-terminal domain, neutralize the virus, and some others give good reaction in immunoblotting or ELISA method [2-4]. The use of these moabs might contribute to further characterization of all MHV isolates. Furthermore, it is known that herpes viruses are able to induce several types of persistent or latent infections and may be under certain conditions oncogenic. In studies of interaction between the various types of host cells and various herpes viruses, a new class of substances resembling growth factors probably encoded by virus genome has been obtained. These factors are produced in certain cells in conditions non-permissive for virus replication, e. g. at supraoptimal temperature or in the presence of inhibitors of DNA replication, or in the cells which are naturally non-permissive for virus replication. Although these factors are only partially purified at present, their fundamental characteristics already exist. The cultivation of non-transformed cells in the presence of these factors leads to the appearance of the transformed phenotype, while the phenotype of transformed cells becomes changed towards the normal ones [5].

At the present, the trends in modern analytical chemistry and biosciences are closely connected with the necessity to solve the complex analytical problems associated with multi-component qualitative and quantitative analyses of substances present in complex biological matrices at too different concentration levels (3-10 decimal orders). For successful solution of analytical problems associated with the analysis of substances present in complex matrices, one is always looking for new analytical approaches, often based on already known high-performance separation and detection techniques.

This work was focused on detailed study of biologically active substances induced murine herpes virus 68 using MS techniques in combination with different separation techniques.

2 Experimental

2.1 HPLC-MS analysis

All LC-MS analyses of our samples were performed by using Shimadzu LC-MS-IT-TOF ™ (Shimadzu, Kyoto, Japan). This MS analyzer is combining an electrospray ionization (ESI), a 3D quadrupol ion trap (IT) and an orthogonally accelerated time-of-flight analyzer (TOF) as providing both the high sensitivity and high resolution of ions. LC experiments were performed on Ascentis C18 column (100/2.1 mm; 5 μm) (Sigma-Aldrich (Steinheim, Germany) as using a gradient elution (water – acetonitrile) with a 0.2 ml/min flow rate: 0-1 min. - 10% ACN, 1-6 min. - 10-90%, 6.01-12 min. - 10% ACN. The column was thermostated to 40°C. The MS conditions were as follows: electrospray capillary voltage +4.5 kV and -3,5 kV in postive and negative ionization mode, respectively. Drying gas flow rate 10 L.min-1, drying gas temperature 200 °C. The MS – MSn experiments automatically acquired data within 100-1000 m/z values in both the positive and negative modes. Data acquisition in MSn mode was done under 25% energy with ion isolation window + 1 m/z. Data acquisition and evaluation was performed using LCMS Solution ver.3.4.151 (Shimadzu). Chemometric evaluation of individual fractions was realized by MSXelerator software ver. 2.4 (MSMetrix, Maarssen, Nederland).

2.2 Chemicals

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 for LC-MS were purchased from Merck (Merck, Darmstadt, Germany). Cell lines: BHK-21, NIH-3T3 and HeLa cells, Virus: MHV-68, MHV-76.

- Titration: the transforming and transformation repressing activity was titrated in BHK-21 cells or in Hela cells respectively.
- RP-HPLC: following separation on the RP-HPLC Separon SGX C18 column 25 fractions tested for biological activity were obtained.
- FPLC: After using phosphate buffer (pH= 7.2) for elution on FPLC Sephadex G15 column, 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.

3 Results and Discussion

The addition of the medium of MHV infected cells cultivated at 41^{o}C resulted in an appearance of the transformed phenotype, the cells aquired the appearance of cells with the loss of contact inhibition. An opposite effect was observed in transformed HeLa cells which acquired the appearance of non-transformed cells. 2 out of 7 monoclonal antibodies (moabs) directed against gB glycoprotein of MHV-68

neutralized both activities mentioned above, however, this neutralization was observed only with medium from MHV-68 and not from MHV-76 infected cells.

Application on RP-HPLC Separon SGX C18 column resulted in appearance of 2 fractions with transforming activity and devoid of transformation repressing activity. No differences between MHV-68 and MHV-76 were observed. Different results dependent on elution solution were obtained with MHV-68-associated factor on FPLC Sephadex G15 column. When phosphate buffer (pH 7.2) was used, the highly active fractions possessing both activities were obtained, while when the column was eluted with redistilled water, the obtained fractions possessed only transformation repressing activity (see Figs.1).

The obtained fractions were analysed using LC-IT-TOF MS analyzer (Shimadzu) in positive and negative ionization modes. We have discovered from the obtained chromatograms and MS spectra that the individual fractions have a different composition. MSXelerator software version 2.4 was used for classification of individual fractions. Implemented data processing enabled us to obtain 2D maps of individual sample profiles. Finally obtained 2D maps significantly eliminate the noise and show only the most important ions presented in the sample .The obtained results allow characterizing and classifying fractions. The fraction obtained by the FPLC separation with phosphate buffer reporting highest biological activity (fraction #6) was used in experiments with solid phase extraction. Because of unknown structure of biologically active substances induced murine herpes virus different types of SPE sorbents a different pH were tested.

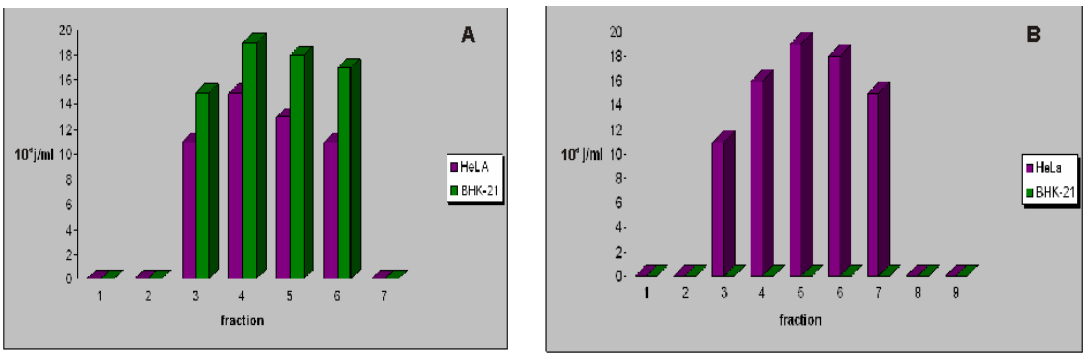

Fig. 1. The biological activity of the fractions obtained by the FPLC separation on the Sephadex G15 column after elution with phosphate buffer (pH = 7.2) A) and with deionized water B).

4 Conclusions

Identification power of LC-IT-TOF MS in combination with chemometric data evaluation was demonstrated and our future experimental work will be focused on detail MSn analyses of the fractions to identify the components responsible for such biological activites.

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P110 ANALYSIS OF AQUEOUS SOIL EXTRACTS BY THIN LAYER CHORMATOHRAPHY

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1 Introduction

This work deals with the study of the possibilities of using thin layer chromatography (TLC) analysis of the aqueous alkalic (NaOH, NH_4OH solution) extracts as a screening tool for the selection of suitable chromatographic systems that can be adapted or otherwise further utilized in high performance liquid chromatography (HPLC) for soil constituents analysis or preparative separation. The aim is to find the easiest and best conditions for selection of mobile and stationary phases for separation of substances in multicomponent soil extracts.

Soil organic matter is classified to humic and non-humic substances. Humic substances are humic acids (HA), fulvic acids (FA) and humin. Non-humic compounds are known biochemical classes: carbohydrates, lipids, amino acids and the others. TLC was chosen as a screening method to get initial knowledge about the complicated multicomponent sample and adopt the information for more expensive analztical or preparative HPLC separation and/or characterization soil sample constituents. TLC is more suitable and less expensive with respect to the nature of the samples. TLC may be complementary technique to some HPLC separation mechanisms and in some cases its separation mode can be also orthogonal to certain HPLC separation mode. Humic acids and non-humic compounds are highly complex

molecules and can cause a number of problems during direct HPLC analysis [1, 2]. Therefore we logically recognized need for adaptation of suitable TLC technique combined with TLC plates scanning and evaluation with suitable generally available image processing software (Origin) for data processing, i.e. approach which is recently seldom used in spite of the fact that all needed for the purpose is commonly available.

In this work is studied the influence of image processing color contrast of scanned TLC plates to the parameters of 16-bit resolution digitized chromatogram (signal to noise ratio) and the impact of various mobile phases composition to separation resolution of various soils constituents of extracts [3].

2 Experimental

2.1 Soil

We studied components of four soil samples: from Slovak localities Skalka, Stupava, Šajdíkové Humence, Gbely, Voderady. The soil samples were pedologically characterized in detail [4]. Soils were extracted by 1M NaOH, 2 M NaOH and NH_4OH, resp. [5, 6].

2.2 Reagents and material

Ammonia solution 28-30%, (Merck, Darmstadt, Germany); EDTA (Merck, Darmstadt, Germany); Ethanol (Lachema, n.p. Brno); Methanol gradient grade (Merck, Darmstadt, Germany); Sodium chloride - NaCl (Lachema, Brno n.p.); Ultrapure water (Simplicity UV, Millipore S.A.S., France)

TLC plates:

Fixion 50x8 Na+, (20 x 20 cm), (Reanal, Budapest, Hungary)

Fixion 2x8 Anion, (Reanal, Budapest, Hungary)

Lucefol (20 x 20 cm)/ (15 x 15 cm), (Cavalier, Czech Republic)

Silufol (20 x 20 cm) / (15 x 15 cm), (Cavalier, Czech Republic)

2.3 Instruments

- PC, Scanner
- OriginPro 8 Software, (OriginLab Corporation, USA)
- Centrifuge Eppendorf AG 22331, (Hamburg, Germany)
- Containers for developing TLC plates

3 Results and Discussion

For digitization of information encoded in the form of spots on the TLC plate into a digital data and its reconstruction into a chromatogram (signal intensity as a function of R_F values), we have used scanned developed TLC plates (25x25cm) with an ordinary scanner with adjusted length resolution of 200 dpi (resolution 0.13 mm) with a 16 bit color depth resolution (true color) in format 1:1. Subsequently scanned images of TLC plates were stored in the bit map format, processed with the aid of program Microcal Origin® Pro 8, which allowed to obtain in the selected directions

data files in the form of dependence of intensity of black shades on the position. The dependence was recalculated to the TLC chromatographic data record. A pair of figures, the intensity vs. position were further processed in the MS Office Excel by evaluation of known relationship $k = (1 /R_F) -1$ for the conversion of R_F values obtained from TLC into retention factor k (as HPLC column equivalent), see Fig. 1. This approach allowed us systematically use and evaluate relatively fast and inexpensive separation conditions for column chromatography even under extreme conditions of pH, ionic strength, composition of aggressive solvent mixtures and so on selected types of stationary phases.

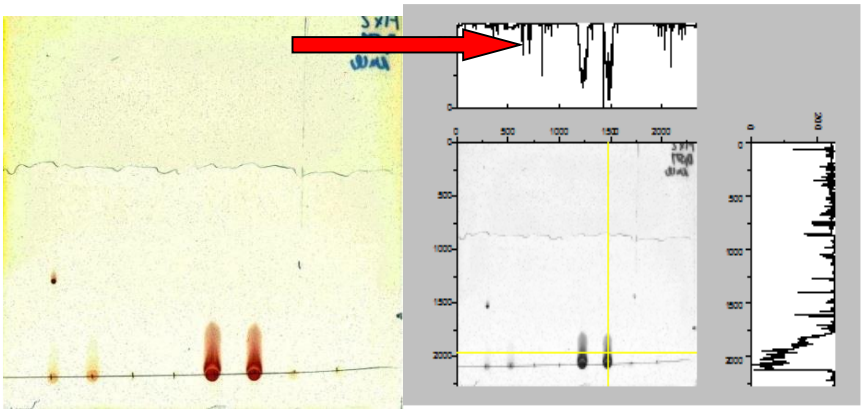

Fig. 1. Principle of TLC chromatogram processing into the digitized chromatogram using some functions of Microcal Origin.

We systematically studied the impact and effect of the composition of the mobile phase and the stationary phase retention of various extracts components of soil samples. The essential part of our research is focused to processing of graphical information from TLC digitized chromatogram. Beside the study a wide range of variables was studied, for example the impact of color corrections, the contrast adjustment of an image when scanning to the signal-to-noise ratio of the digitized chromatogram, as is shown in Figure 2.

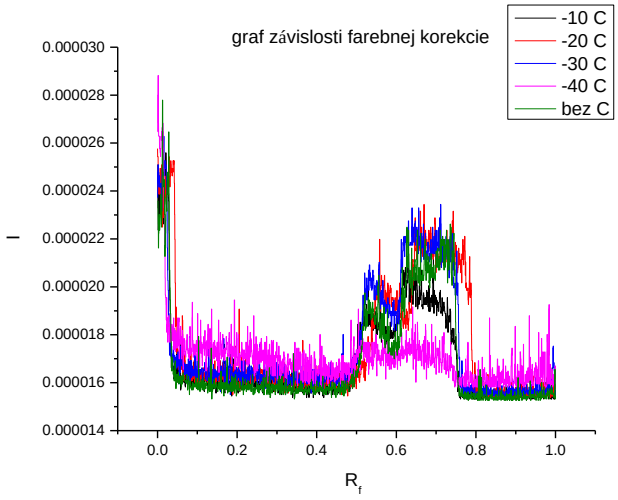

Fig. 2. Impact of color correction by the image contrast of TLC (Lucefol) separation of soil Šajdíkové Humence extract (1 mol.L^{-1} NaOH, mobile phase 0,1 mol.L^{-1} NaCl in water).

4 Conclusions

This work dealt with the methodology of selection of appropriate conditions for analysis of soil organic matter by thin layer chromatography as a preliminary optimization method before HPLC analysis of complex soil extracts.

Program Origin has proved to be helpful in evaluating data. From the plot follows that the best color correction is without modification however, for better visualization it is better to choose correction of -20 C.

Acknowledgement

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P111 ULTRA-FAST ONLINE SPE-MS/MS METHOD FOR QUANTIFICATION OF FOUR TYROSINE KINASE INHIBITORS IN HUMAN PLASMA

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1 Introduction

Tyrosine-kinase inhibitors (TKIs) are class of anti-cancer drugs used to treat various forms of blood cancers or solid tumors. To individualize daily dosage, diagnosing of undertreatment or avoiding toxicity, therapeutic monitoring of TKIs in patients plasma could be advantageous [1]. We assessed the performance of an ultra-fast online SPE-MS/MS system (RapidFire) to analyze four TKIs, Imatinib (IMA), Dasatinib (DAS), Nilotinib (NIL) used for treatment of e.g. chronic myeloid leukemia and Lapatinib (LAP), that is used against breast cancer and other solid tumors, in human plasma. All four analytes are measured within wide calibration range in less than 15 sec, providing high throughput and savings in solvent consumption, with analytical results comparable to traditional LC-MS methods [2].

2 Experimental

Calibration standards were prepared by spiking blank human plasma with four TKIs to final concentrations of 0.01 – 10 µg/mL for NIL and LAP, 0.03 – 10 µg/mL for IMA and 0.025 – 5 µg/mL for DAS. Calibration standards, quality control samples and samples from patients treated with TKI were then precipitated by methanol containing internal standards (deuterated TKIs). After centrifugation (14,500 × g; 5 min), supernatant was diluted 10 times with water. Samples were analyzed at a rate 14.5 seconds per sample on a RapidFire ultra-fast autosampler/in-line SPE system (Agilent Technologies, Lexington, MA) coupled to a QTRAP 5500 triple quadrupole instrument (AB Sciex, Foster City, CA, USA). C18 column was used for SPE and elution was done by mixture of acetonitrile:isopropanol (1:1) containing 0.1% formic acid. Analytes and their internal standards were monitored in positive MRM mode. RF*Integrator* software was used for data analysis.

3 Results and Discussion

To establish intra- and inter- day precision and accuracy, prepared calibration standards and quality control samples were run in triplicate over a series of days. All compounds analyzed had both intra- and inter-day accuracies within 15% and coefficient of variation less than 15% for all concentrations within the linear range. Isopropanol was added to acetonitrile as an eluent to fully elute all compounds from SPE C18 column. Carry over was assessed by running a blank sample immediately after high calibration point and was less than 1% for all TKIs. To completely avoid any potential carry over we recommend using a single blank solvent injection for high concentrations. Matrix effects, evaluated by comparison of samples prepared in neat solution, and in five different lots of human plasma, were 70±37%, 86±26%, 101±28% and 74±33% for IMA, NIL, LAP and DAS respectively. Because of deuterated internal standards use, the matrix effects were eliminated.

4 Conclusions

A single 14.5s SPE-MS/MS method was developed for analysis of four TKIs in plasma with results comparable to standard LC-MS methods. This system allows throughput of > 240 samples per hour without significant carry over. With increasing number of patients receiving TKIs, this method can be advantageously used for high-throughput therapeutic monitoring of these drugs in clinical research laboratories.

Acknowledgement

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P112 ISOTACHOPHORETIC DETERMINATION OF KETONE BODIES AND LACTATE IN CATTLE SERUM

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Summary
The aim of the work was the optimization of isotachophoretic determination of lactate and ketone bodies (β-hydroxybutyrate and acetoacetate) for veterinary medicine. The measurement of these metabolites is very important for the evaluation of metabolic diseases of cattle. The complete separation of these three analytes together with other possible blood components was achieved using 10 mmol/l hydrochloric acid with β-alanine as a leading electrolyte at pH = 3.15 and 10 mmol/l sodium propionate as a terminating electrolyte. Limits of detection determined from the standard mixture for acetoacetate, lactate and β-hydroxybutyrate were 3.75 µmol/l, 3.96 µmol/l and 7.46 µmol/l, respectively.

1 Introduction

Metabolic diseases and especially ketosis represents a serious problem in dairy cattle. The importance of ketosis is primarily in reduction of milk production, changes in milk composition and disruption of reproductive functions.

For proper management of ketosis in farms the precise diagnosis and evaluation of metabolic state of the animals is essential. Ketosis is characterised by increased concentration of ketone bodies (β-hydroxybutyrate, acetoacetate and acetone) in blood, which are synthesised by the liver due to inadequate amounts of oxaloacetate. In ruminants oxaloacetate is used preferentially for gluconeogenesis and thus the capacity of the liver for oxidation of fatty acids is limited. The production of ketone bodies by the liver in ruminants is more common than in other species of animals. Increased amounts of β-hydroxybutyrate and acetoacetate in blood cause metabolic acidosis, which can be complicated also by increased concentration of lactate. In ruminants, the important source of lactate is rumen microflora beside anaerobic glycolysis in tissues. During metabolic acidosis the concentrations of ketone bodies and lactate in blood are increasing. The amount of each acid is influenced by the etiology that is necessary to take into account during therapy of the patients.

Nowadays ketone bodies are usually determined in clinical practice by the enzymatic oxidation of β-hydroxybutyrate to acetoacetate accompanied by indirect photometric detection of reduced NADH. Analogous principle is used for lactate detection. In last years electronic handheld blood analysers for measurement of

β-hydroxybutyrate and lactate are used as well [1], but these methods are expensive and those usage is limited. Ketone bodies can be determined by GC-MS [2] or HPLC after precolumn derivatization [3].

For determination of ketone bodies together with lactate and other blood components isotochophoresis can be also used successfully [4]. It is a low-cost method where more organic acids can be determined in one analysis which helps to the precise diagnosis and therapy of the animals. In this paper the optimized method for determination of ketone bodies (β-hydroxybutyrate, acetoacetate) and lactate was applied to cow serum.

2 Experimental

Capillary isotachophoresis was performed on Electrophoretic Analyser EA 303A (Villa Labeco, Slovakia) at an ITP-ITP mode. As a leading electrolyte 10 mmol/l hydrochloric acid with β-alanine was used; the pH of this solution was optimized. 10 mmol/l sodium propionate served as a terminating electrolyte. The current was set to 350 μA in the first capillary with ID 800 μm and to 60 μA in the second capillary with ID 300 μm. Serum samples were diluted 10 times by distilled water prior the analysis.

3 Results and Discussion

When optimizing the working conditions, we took into account also other possible serum components. Citrate, α-hydroxybutyrate and acetate where chosen to complete the standard mixture. Two analytes, acetoacetate and lactate, were separated well when glutamic acid served as terminating electrolyte. The third analyte of interest, β-hydroxybutyrate, was not differentiated due to the low effective mobility, thus the usage of propionic acid as a terminating electrolyte was necessary.

The previously published pH value of 3.3 showed poor separation of acetoacetate and citrate under our conditions. Higher pH did not improve the situation and at pH 3.75 there was generally no separation of the components. Lower pH showed better resolution and finally the pH value was set to 3.15, where all the analytes of interest together with other possible serum components were fully separated. Also the citrate and acetoacetate showed weak but clear resolution (Fig. 1). Acetate migrates behind β-hydroxybutyrate although its effective mobility is higher and creates a zone positioned below the zone of butyrate. This effect is called enforced isotachophoresis and it is typical for acetate. Limits of detection determined from the standard mixture for acetoacetate, lactate and β-hydroxybutyrate were 3.75 μmol/l, 3.96 μmol/l and 7.46 μmol/l, respectively.

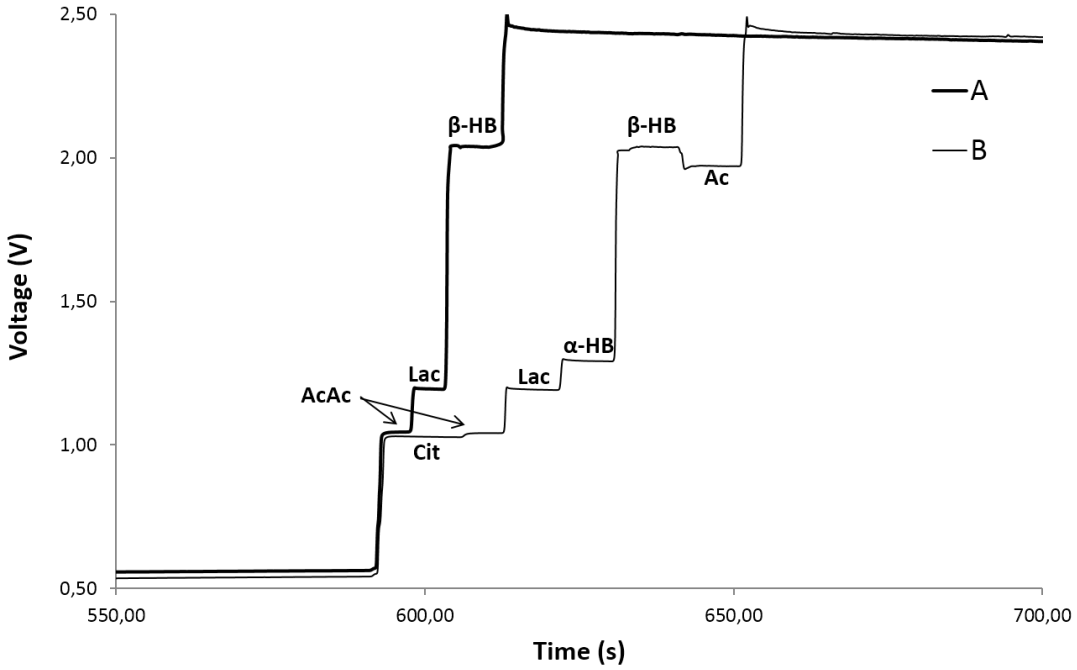

Fig. 1. Isotachophoretic separation of standard mixture. A - 3 analytes (AcAc – acetoacetate, β-HB - β-hydroxybutyrate and Lac – Lactate); B – 6 analytes (additional: Ac – acetate, Cit-citrate and α-HB - α-hydroxybutyrate).

The optimized method was applied to serum of healthy as well as ill cows. The values of β-hydroxybutyrate and acetoacetate reached up to 7.83 mmol/l and 0.54 mmol/l, respectively. The values of β-hydroxybutyrate corresponded well with the results previously determined by the photometric method. Lactate concentration strongly depends on the way of blood processing. If fluoride is added into the sample, the glucose metabolic pathway is stopped and the lactate concentration remains stable. The lactate values in our samples reached up to 2.63 mmol/l. On the other hand if there is no fluoride addition, the glycolysis keeps going and the lactate concentration increases rapidly up to 10 mmol/l. The difference between these two types of storage is shown in the Fig. 2.

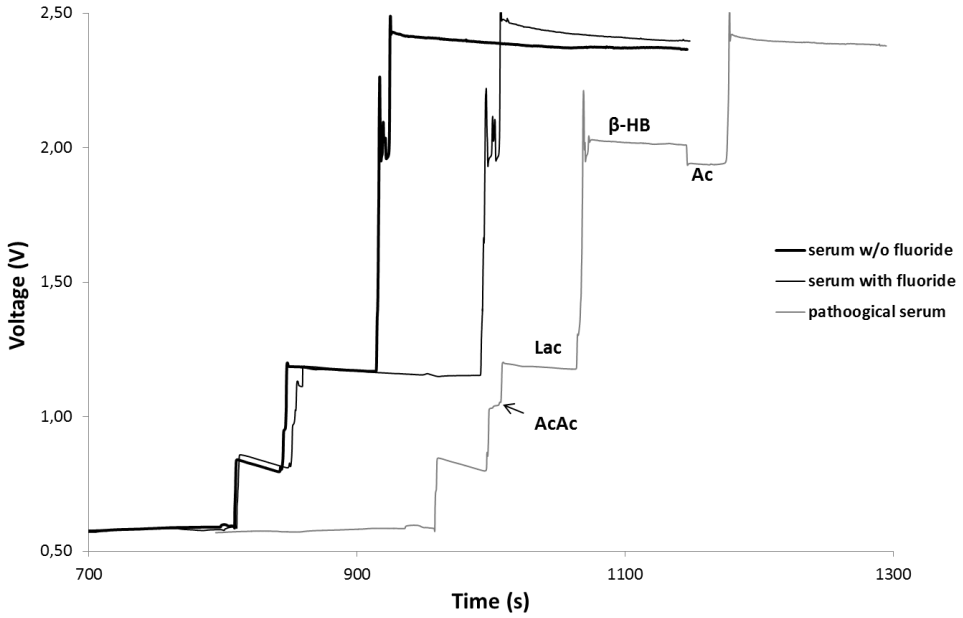

Fig. 2. Isotachophoretic separation of cow serum (Ac – acetate, AcAc – acetoacetate, β-HB - β-hydroxybutyrate and Lac – Lactate).

4 Conclusions

Isotachophoretic determination of ketone bodies or other organic acids can be used as a routine, low-cost, fast and effective method in analysis of blood or different biological materials. Thus it can help to better and precise diagnosis of metabolic diseases in cattle.

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P113 CELLULASE IMMOBILIZATION ON CARRIER FROM WASTE POLYETHYLENE TEREPHTHALATE BOTTLES

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Summary

Cellulase enzyme of *Trichoderma* was immobilized by using crushed polyethylene terephthalate (PET) bottles obtained from wastes as carrier. The properties of the

immobilized cellulase were investigated such as the thermal stability, storage and operational stability and the mode of action of the enzyme on the polymeric substrate. The obtained results were compared with free enzyme and two other commercial carriers (Sorsilen, Eupergit C).

1 Introduction

Cellulase, a multi-component enzyme consisting of three different enzymes (endocellulases, cellobiohydrolase and β-glucosidases), is responsible for the bioconversion of cellulose into soluble sugars. Cellulases are used mainly in the pulp, paper, textile, food industries and also for biofuel production [1]. The main reason for using an enzyme in an immobilized form is that it provides better handling of the enzyme and its easy separation from the product which facilitates efficient recovery and reuse of costly enzymes. And it also minimizes or eliminates protein contamination of the product. With immobilization one can achieve continuous, fixed-bed operation and it often enhances the stability [2]. In the present work, the crushed PET bottles were used for the first time as enzyme carrier for the cellulase immobilization. This carrier is effective, environmental-friendly (reuse of waste) and economical alternative for many of the current, commercial and expensive carriers available. It can also be used in the food industry as it does not contain any harmful or banned substances.

2 Experimental

2.1 Immobilization of cellulase

1 g of carrier suspended in a 0.05 mol/L acetate buffer of pH 4.8 (20 ml) was incubated with the enzyme (lyophilized cellulase from *Trichoderma reesei* ATCC 26921, Sigma-Aldrich) at 4 °C under stirring for 48 h. The carrier was centrifuged and repeatedly washed with the buffer until the absence of non-adsorbed enzyme was confirmed in the supernatant. The immobilized enzyme was then stored in an acetate buffer of pH 4.8 in form of a suspension. The same procedure was used for the preparation of immobilized enzymes on Sorsilen and Eupergit C.

2.2 Enzyme assay

The enzyme activity was measured by the carboxymethylcellulase activity method [3]. Activity of free and immobilized enzyme was determined at pH 4.8 (0.05 mol/L acetate buffer) and 25 °C. Incubation of the reaction mixture with immobilized enzyme was performed under constant stirring in a constant-temperature double-jacketed vessel. The operational stability of the immobilized enzyme was examined by continuous washing of the column by CMC solution (0.25 %, pH 4.8) at 3.5 mL/h flow rate, at room temperature. Reducing groups in the eluate were periodically tested. The stability of immobilized enzymes, stored in acetate buffer of pH 4.8 at 4 °C, was monitored once a week. The heat stability was characterized on the basis of activities determined after 2 h-incubation of the enzyme at the employed temperature (25 – 80 °C) and followed by cooling to 25 °C. Mode of action of free and immobilized enzyme was determined by viscometry. Measurement of viscosity

decrease during the degradation of a substrate (0.8% CMC) was realized in Ostwald viscometer at 30 °C, the concentration of reducing sugars was determined simultaneously.

3 Results and Discussion

3.1 Operational stability

For PET carrier the activity remained constant after a significant decrease during the first 48 hours of the process. The steady state was reached at around 30 % of residual activity, and it lasted next 20 days (Fig. 1 A). The operational stability is as good as of enzyme immobilized on Eupergit C and much better than of enzyme on Sorsilen.

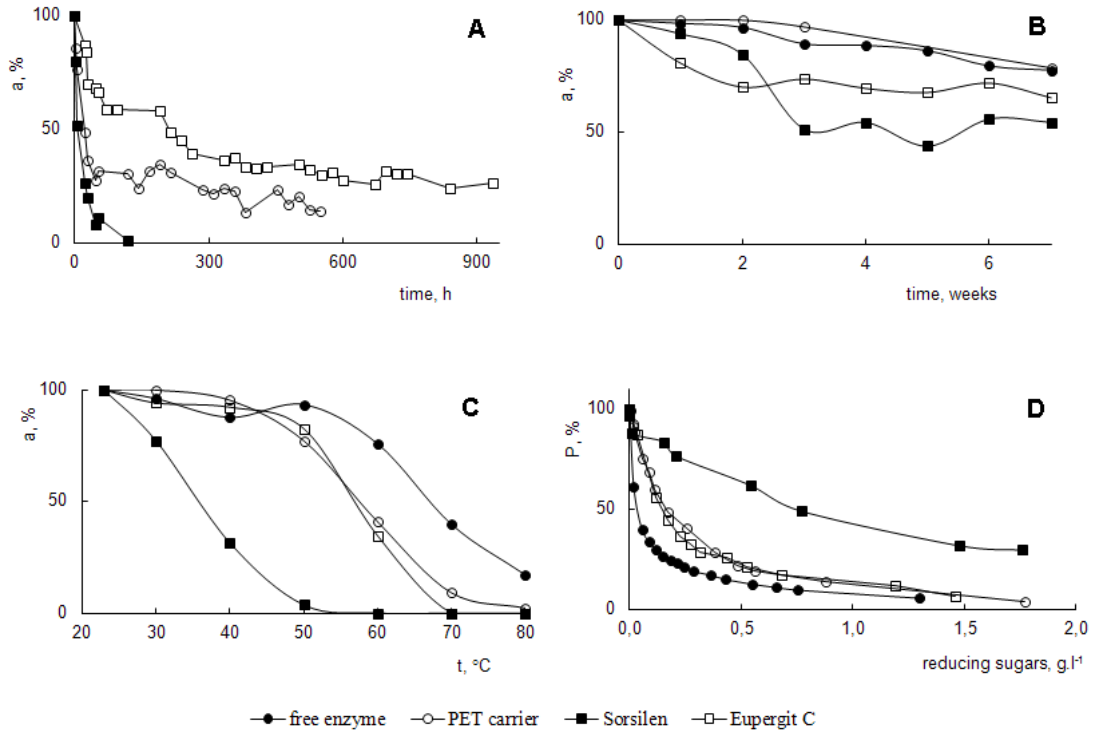

Fig. 1. (A) Operational stability of the enzyme immobilized on various carriers, (B) Storage stability of the immobilized and free enzyme, (C) Thermal stability of the immobilized and free enzyme, (D) Dependence of viscosity decrease on reducing groups.

3.2 Storage stability

Within the above mentioned conditions, the activity of Sorsilen prepared decreased during the first three weeks by almost 50 %; in the case of Eupergit C, it decreased during the first two weeks by 30 %, while preparation of PET remained stable for first two weeks and decreased after 7 weeks by merely 25 % (Fig. 1 B). The decrease in the activity was not associated with the liberation of the enzyme from the carriers.

3.3 Thermal stability

Thermal stability of the enzyme on all the three carriers was found to be low when compared to the free enzyme. PET carrier and Eupergit C showed similar behaviour

as the stability remained unaffected till 50 °C and then decreased regularly. Sorsilen proved to be thermally very unstable, showing no activity at 50 °C (Fig. 1 C).

3.4 Mode of action

Immobilization on the PET carrier or a covalent bond to Eupergit C did not cause significant changes in the degradation of the polymeric substrate (Fig. 1 D). As well as free enzyme, these immobilized enzymes cleave the polymer substrate in a random manner, first applying an endoglucanase which cleaves the substrate to lower oligosaccharides, whereas dimers and monomers are formed at the end of the reaction.

4 Conclusions

The above results showed that the proposed immobilization of cellulase on PET carrier was satisfactory for further applications. The storage stability remained very high and it also showed high operational stability enabling long-term continuous use. Mode of action of the enzyme is not changed by immobilization on this carrier. Thus it can be an economical alternative to commercial and expensive carriers.

Acknowledgement

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