THESIS ON NATURAL AND EXACT SCIENCES B101

New approaches in capillary electrophoresis for separation and study of proteins

TATJANA KNJAZEVA

TUT PRESS

TALLINN UNIVERSITY OF TECHNOLOGY
Faculty of Science
Department of Chemistry

Dissertation was accepted for the defence of the degree of Doctor of Philosophy in Natural and Exact Sciences on January 14th, 2011

Supervisor: Professor Mihkel Kaljurand, Department of Chemistry, Faculty of Science, Tallinn University of Technology, Estonia

Opponents: Professor Boguslaw Buszewski, Chair of Environmental Chemistry and Bioanalytics, Faculty of Chemistry, Nicolaus Copernicus University, Poland

Professor Ursel Soomets, Department of Biochemistry, Medical Faculty, University of Tartu, Estonia

Defence of the thesis: March 17th, 2011

Declaration:

Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any academic degree.

Tatjana Knjazeva

ISSN 1406-4723
ISBN 978-9949-23-060-0

LOODUS- JA TÄPPISTEADUSED B101

Kapillaarelektroforeesi uued lahendused valkude lahutamisel ja uurimisel

TATJANA KNJAZEVA

TTÜ
KIRJASTUS

Моей семье посвящается

CONTENTS

LIST OF PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals.

I. T. Knjazeva, M. Kulp, M. Kaljurand. CE separation of various analytes of biological origin using polyether ether ketone capillaries and contactless conductivity detection. *Electrophoresis 2009, 30, 424-430*.

II. K. Helmja, M. Borissova, T. Knjazeva, M. Jaanus, U. Muinasmaa, M. Kaljurand, M. Vaher. Fraction collection in capillary electrophoresis for various stand-alone mass spectrometers. *J. Chromatogr. A 2009, 1216, 3666-3673*.

III. T. Knjazeva, M. Kaljurand. Capillary electrophoresis frontal analysis for the study of flavonoid interactions with human serum albumin. *Anal. Bioanal. Chem. 2010, 397, 2211-2219.*

THE AUTHOR'S CONTRIBUTION TO THE PUBLICATIONS

I. The author planned and carried out all the experiments. She interpreted the results and wrote the major part of the manuscript.

II. The author optimized the experimental setup and performed the practical work concerning the CE-MALDI-TOF-MS approach. She discussed the results and participated in writing the manuscript in collaboration with the co-authors.

III. The author was responsible for planning and performing the experimental part. She interpreted the data obtained and wrote the manuscript.

ABBREVIATIONS

ACE	affinity capillary electrophoresis
BGE	background electrolyte
C4D	capacitively coupled contactless conductivity detection
CE	capillary electrophoresis
CE-FA	capillary electrophoresis frontal analysis
CGE	capillary gel electrophoresis
CIEF	capillary isoelectric focusing
CZE	capillary zone electrophoresis
ELISA	enzyme-linked immunosorbent assay
EMMA	electrophoretically mediated microanalysis
EOF	electroosmotic flow
ESI	electrospray ionisation
His	histidine
HIV	human immunodeficiency virus
HPLC	high-performance liquid chromatography
HSA	human serum albumin
ICP-MS	inductively coupled plasma mass spectrometry
IR	infrared spectroscopy
K	binding constant
LED	light emitting diode
LOD	limit of detection
MALDI	matrix-assisted laser desorption/ionisation
MEKC	micellar electrokinetic chromatography
MES	2-(N-morpholino)ethanesulfonic acid
MS	mass spectrometry
n	number of binding sites with the same affinity per protein
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEEK	polyether ether ketone
PMMA	poly(methyl methacrylate)
PTFE	polytetrafluoroethylene
PVA	poly(vinyl alcohol)
r	number of total ligands bound per protein
RP	reversed phase
RSD	relative standard deviation
SD	standard deviation
SDS	sodium dodecyl sulphate
TFA	trifluoroacetic acid
TOF	time of flight

INTRODUCTION

The development of efficient and sensitive analytical methods for the separation, identification and quantification of various analytes of biological origin is continuously a topic of high interest for a broad spectrum of sciences. Among the many branches of biochemistry no other field except proteomics has attracted such numerous technological and financial facilities over the past decade. The intense interest in proteins is largely driven by their essential role in numerous biological processes and was particularly accelerated by a recent success of the human genome project. Meanwhile, any progress in proteomic research is rather dependent on the advances in analytical tools. Therefore, the challenging tasks of large-scale proteomics have necessitated the designing of novel instruments and planning alternative research strategies as well as the improving of existing techniques.

Among the basic requirements for analytical techniques the high efficiency and sensitivity in the wide dynamic range of proteomic separations with minimal sample consumption are the most important. Traditionally, the two-dimensional polyacrylamide gel electrophoresis has been a "workhorse" technique for protein separation, whose superior resolving power, however, is overshadowed by the poor sensitivity and reproducibility of results. Nowadays, mass spectrometry (MS) is a fundamentally important analytical tool in proteomics that is capable of the identification and quantification of proteins and characterization of their modifications. While the direct MS measurements of simple mixtures are feasible, the great complexity of natural and biological samples stresses the enormous need for using a supplementary separation method prior to MS analysis.

Capillary electrophoresis (CE), especially in combination with a highly sensitive MS detection, has become a promising analytical technique in proteins study. Currently, hyphenation with MS is still on the development stage in spite of the many technical improvements in design proposed in the past years. The most challenging point of an on-line coupling is the interface between CE and MS. Therefore, alternative off-line approaches by electrophoresis are considered convenient for a further study with appropriate stand-alone spectroscopic techniques. Generally, the versatility and simplicity of an electrophoretic instrument renders the CE technique advantageous over traditional methods. Indeed, the diversity of detection modes and availability of new capillary materials with different surface properties, unlike fused-silica, enables a significant increase of method applications, with protein separation included. Moreover, a complete instrument can be miniaturized to a high extent from electronics up to detector resulting in relatively compact systems. As a result, a variety of innovative instrumental platforms from CE-based portable devices for in-field analysis up to multidimensional systems are available nowadays for an extensive study of proteins. Furthermore, the potential of CE for an automated, fast and high-efficient analysis makes it feasible to be a complement to conventional techniques or even a replacement for some labor-intensive assays.

Likewise, of special interest has been the application of CE to the characterization of interactions between target proteins and numerous biologically important molecules. This is attractive for proteomic and pharmaceutical researches, where understanding of interactions is essential for the control of a large number of processes in biosystems.

On the whole, proposed initially as a tool for the analysis of small charged molecules, CE is nowadays used for the study of proteins, which seems to be one of the most impressive fields of application of the technique. Although the method is diversely realized, novel approaches may be still further explored. Thus, the advanced development of the CE-based analysis with main emphasis on the protein research was aimed and investigated in the present work.

AIMS OF THE STUDY

The main goals of the present thesis were to demonstrate the various new opportunities that capillary electrophoresis opens for protein analysis. The novel approaches were essentially focused on the development and description of proposed systems as well as on the demonstration of advanced applications.

More specifically the aims set were:

- to investigate the utility of the polyether ether ketone (PEEK) capillary for CE separation with contactless conductivity detection
    - to develop simplified CE protocols for the analysis of proteins, peptides and other analytes of biological origin
    - to compare the separation performance of the polymer tubing with that of a traditional fused-silica capillary on model sample mixtures
- to present a CE fraction collection system for various stand-alone mass spectrometric instruments as a means of compound identification, MS profiles simplification and interfering species removal
    - to design and discuss the capillary electrophoresis fraction collection system for an off-line MS combination
    - to validate the off-line CE-MALDI-TOF-MS approach to proteins analysis
- to apply capillary electrophoresis frontal analysis (CE-FA) to a comprehensive characterization of protein-ligand binding properties
    - to determine binding constants on an example of human serum albumin and structurally different flavonoids
    - to consider the possible relationship between the binding capability and structural specificities of the compounds under study
    - to propose a new extended application for CE-FA method to identify a specific binding site and characterize the predominant intermolecular forces in studied complexes

1 LITERATURE OVERVIEW

1.1 Analytical tools for proteomic research

After realization of the Human Genome Project the main attention of bioanalysts was concentrated on the next challenging task as protein analysis. Indeed, proteins are complicated biomolecules that are made up of 20 naturally occurring amino acids and are folded into a very well-defined form, which essentially affects their function and activity. Additional posttranslational modifications more amplify the structural complexity. Moreover, like other biological macromolecules, proteins are an important part of organisms and participate in many processes within the cells: catalyze specific biochemical reactions, have structural and transport functions, important for cell signalling and immune responses[1,2]. In contrast to genome, proteome is dynamic over time and differs depending on the variety of physiologies and environments. Taking into account the diversity of biological systems the study of thousands of peptides and proteins at a concentration range of over several orders of magnitude represents a significantly more formidable task than the genome research.

All extensive investigations of protein species, particularly their structure and biological role, are associated with the term *proteomics*[3,4] (Figure 1). The first studies in the field were mainly focused on simple measurements of protein content, but advances in the instrumentation and methodologies allowed an expansion of the scope of biological studies to the analysis of complex protein samples. At present, the discovery and utilization of specific protein biomarkers for a quick diagnosis of particular diseases is one of the most promising applications of proteomics[5-8]. Moreover, systematic studies imply the structural analysis and ascertainment of the function of each protein, as well as the understanding of protein interactions in complex networks and pathways. On the whole, the ultimate aim is to completely map the proteome[3].

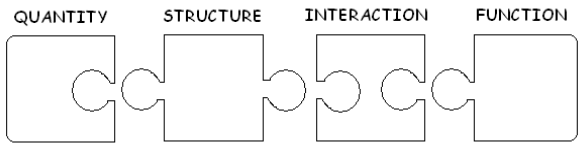

Figure 1. Pieces of the proteomics puzzle

Scientists are evidently faced with a variety of tasks in the proteomic area. Nevertheless, a wide scope of techniques from classical analytical chemistry is now available for proteome research. The potential of new developments to assist in the analysis of proteins is also used. Since a proteomic studies can be subdivided into the several disciplines, the role of each technique is clarified after the problem setting:

· Qualitative and quantitative estimation of proteins

In the simplest way, the determination of proteins can be performed by spectrophotometry owing to the characteristic absorption maximum of the peptide bond around λ = 205 nm and the aromatic ring of amino acids tryptophan and tyrosine around λ = 280 nm[1,9,10]. Additionally, the Biuret test, Lowry and Bradford assays are traditional colorimetric approaches to measuring the total level of protein in a sample. The methods are based on the reaction of proteins with specific reagents followed by measurement of colour intensity, which is directly proportional to the protein concentration[9-11].

However, the proteomic research often aims at investigating one certain or all individual proteins in the sample. Thus, separation techniques are more advantageous over the preceding since proteins can be measured directly on-column during the separation process[1,12,13]. Among the methods a high-performance liquid chromatography (HPLC)[14], and polyacrylamide gel (PAGE)[1,15,16] and capillary[17,18] electrophoresis are more often used for the qualitative and quantitative analysis of mixtures, as well as for sample purification. Nevertheless, the most powerful systems for analysis of proteins in complicated samples are by far liquid separation techniques coupled to the mass spectrometry in the on-line or off-line modes[19-21].

The distinctive feature of some biomolecules to exhibit a molecular recognition is successfully exploited in such analytical tools as immunoassays and biosensors[1, 22,23]. These approaches are unique in the ability to perform a highly specific and sensitive analysis of the target protein. For instance, the enzyme-linked immunosorbent assays (ELISA) involve enzymes to detect an antibody or an antigen in samples in immunology[24,25] (e.g. food allergens), medicine[26,27] (e.g. HIV test) and various industries[28,29] (e.g. potential toxins, herbicides). Moreover, biosensors can be employed when the protein concentration in the sample is very low and/or the isolation procedure from a complex matrix is a difficultly achieved task. For some practical applications the use of such disposable portable biosensors seems to be preferred to laboratory-based techniques. The weak point of bioassays is still a limited number of applications.

· Structure determination

Currently, the major effort in proteomic analyses is tended to the protein identification and structure determination. Structural analysis is quite a labour-intensive and time-consuming process since it involves a number of reactions and analysis steps to be carried out. In general, after a specific degradation of protein the small fragments obtained are sequenced and the amino acid composition is finally put together[1,30].

The first approaches required an additional chromatographic or electrophoretic separation of cleavage fragments for their further individual sequencing, predominantly by the Edman degradation. This is a reliable process for a relatively short peptide sequencing, which is based on sequentially removing the N-terminal amino acid residue in the controlled mode on the fully automated instrument[1]. More recently, proteins were identified by first partially

digesting them by an enzyme (e.g. trypsin, pepsin) and then measuring the characteristic fragments of the digest by a mass spectrometer. The cleavage pattern is identified by comparison against protein sequence database or predicted reference peptide masses[20,31]. The techniques which are particularly available for such analyses are the matrix-assisted laser desorption ionisation (MALDI) time-of-flight (TOF) mass spectrometry and electrospray ionisation mass spectrometry (ESI-MS). Both methods are able to determine molecular weights with high accuracy and provide structural information. The amino acid sequence for an unknown protein can be determined using a tandem mass spectrometer (e.g. ESI-MS/MS)[20,31,32].

The structure of pure proteins can be investigated by nuclear magnetic resonance (NMR)[33] and infrared spectroscopy (IR)[34]. Moreover, electron microscopy[35,36], X-ray crystallographic[30,37] and NMR[30] measurements with the aid of computational approaches propose a three-dimensional structure of proteins.

One of the main challenges in proteomics research is the determination of isoforms and posttranslational modifications. These have an effect on the molecular mass and net charge of the protein and therefore can be characterized using a separation techniques, MS or MS/MS analyses[15,32,38].

· Interactions and functional analysis

A functional analysis has been mainly focused on the investigation of the essential property of proteins to exhibit enzymatic activity. Due to intensive studies the range of assays proposed is vast, but all are principally based on the measurement of either the consumption of substrate or formation of product over time. Traditionally, there are spectrophotometric, fluorometric and radiometric assays, calorimetry and separation methods (e.g. CE, HPLC, thin layer chromatography)[39,40].

The characterization of binding properties has found a broad application in the pharmaceutical science for new drug development and investigation of biologically active compounds, because their overall distribution and intensity of physiological actions is correlated with affinities towards plasma proteins[41]. Moreover, the study of protein-protein and protein-ligand interactions is important for understanding the molecular processes in the cell and is particularly critical for describing the signalling pathways and regulation mechanisms of biological systems. In this context, several analytical approaches have been proposed, including spectroscopic methods (fluorescence, NMR), affinity electrophoresis, surface plasmon resonance and equilibrium dialysis related techniques[42,43].

Depending on the application focused, a more preferred instrumental platform, selective approach or combination of techniques can be employed. Commonly, the criteria of choice depend on the level of the sensitivity and accuracy required, availability of appropriate equipment and reagents, presence of interfering compounds, cost of analysis, ground of convenience, *etc*. Since biologic fluids are mostly very complex, the techniques employed should be

robust and provide excellent resolution as well. From a practical point of view, assays must be easy to perform, automated and low-priced if laboratories process daily a large number of samples.

Most of the methods used to carry out studies of proteins are not ideal or have definite limitations for their analysis. In view of these facts, versatile techniques which, in addition to separation performance, can estimate rapidly and accurately a number of biological parameters are greatly welcomed. In comparison with new opportunities traditional analytical techniques long ago proved to be reliable and robust. Moreover, they have constantly undergone technical improvements demonstrating potentialities inherent to protein analysis.

In this context, the utility of the CE method is frequently overlooked by proteome researchers. Electrophoresis has been gained popularity in the '80s of the last century as a high-throughput technique for DNA sequencing. The later extensive development of the method was accompanied by a continuous concurrence with other techniques and a number of its avowed achievements were wrongly overshadowed. Nevertheless, it is apparent that CE has not exhausted all its possibilities yet. Nowadays, novel strategies and recent developments are opening new opportunities for the application of CE to the analysis and characterization of proteins.

1.2 Capillary electrophoresis

Among separation techniques, capillary electrophoresis is the most universal one and has proved to be suitable for the analysis of both charged and neutral species, ranging from small metal ions to large biomolecules. Almost all proteomic applications can be accomplished using CE as well. The wide prevalence of method follows from the existence of its different modes, which have been developed and adapted to certain purposes. Nowadays capillary electrophoresis is a well-established technique in analytical laboratories around the world and can be a researcher’s first choice owing to highly efficient separation within a short analysis time on the instrument of moderate cost with minimal reagent and sample consumption. These advantages have led to a tool that has excellent capabilities for solving challenging biological problems, including protein analysis.

1.2.1 Basic principles and theory

The formal definition of electrophoresis describes it as the movement of electrically charged species in a conductive media under the influence of an electric field[1,44]. In practical terms, the efficient separation is affected by two main factors: the electrophoretic mobility of individual analytes and the electroosmotic flow (EOF) of the bulk solution.

The *electrophoretic mobility* (μ_{ep}) is a specific characteristic of a compound under the given experimental conditions. It results from the equilibrium between the electric force, which is exerted in the electric field acting in favour

of the ion motion, and the frictional force resulting from the solution viscosity. The electrophoretic mobility is commonly used to describe the migration of ions and is expressed as the ratio of the electrophoretic migration velocity (v_{ep}) over the electric field strength (E):

$$\mu_{ep} = \frac{v_{ep}}{E} = \frac{q}{6\pi r \eta} \tag{1}$$

where q is the net charge of the analyte, r is Stoke's radius and η is the buffer viscosity.

It is evident from the equation that differences in properties such as net charge and/or size of molecules result in different electrophoretic mobilities and provide the basis of the separation mechanism in CE.

The second fundamental factor that affects the migration of compounds is the *electroosmotic flow*. This phenomenon describes the bulk movement of the electrolyte solution through the capillary and is characterized by the electroosmotic mobility (μ_{EOF}) as defined by the following:

$$\mu_{EOF} = \frac{v_{EOF}}{E} = \frac{\varepsilon \zeta}{4\pi\eta} \tag{2}$$

where ε is the dielectric constant of the buffer solution and ζ is the zeta potential. Experimentally, the velocity of EOF can be estimated by measuring the migration time of a neutral marker (e.g. mesityl oxide, acetone or benzyl alcohol) over a fixed distance of the capillary.

The electroosmotic flow originates from the surface chemistry of the inner wall of the capillary. In the fused-silica capillary, numerous silanol groups (Si-OH) become dissociated to silanate ions ($Si\text{-}O^-$) if the pH value of the buffer rises above 3. Formed under these conditions the negative surface charges are compensated by cations attracted from the buffer solution. These cations are arranged into the double layer: the *Stern layer* is rigid and is accumulated close to the negatively charged wall, while the *diffuse layer* extends into the bulk solution and remains slightly mobile. The potential across the layers is called the zeta-potential (ζ). When the electric field is applied, the cations forming the diffuse layer are attracted towards the cathode and the bulk solution is pulled along with them. This leads to a phenomenon called *electroosmotic flow*[1,45]. Polymeric materials exhibit the EOF resulting probably from the adsorption of charged buffer components into the capillary wall[46,47].

According to previous statements, the apparent mobility of each ion could be expressed as the sum of the ion's electrophoretic mobility and the mobility of the electroosmotic flow:

$$\mu_{ap} = \mu_{ep} + \mu_{EOF} \tag{3}$$

As a result, negatively charged analytes move towards positively charged anode in the opposite direction to the EOF flow, while positively charged analytes advance to the cathode additionally accelerated with the EOF. The uncharged analytes move at the same velocity as the electroosmotic flow. Often,

the EOF is predominant over the electrophoretic mobilities of analytes allowing separation of positive, neutral and negative species in the same run.

There are certain strategies to achieve resolution and influence the efficiency of the CE separation process. Fundamentally, the electrophoretic mobility of most compounds is strongly dependent on the pH of the buffer, affecting their ionization. On the other hand, the direction and velocity of the electroosmotic flow depend on the capillary surface charge and buffer parameters. Thus, simple on implementation possibilities to modify EOF are changing the ionic strength and pH of the background electrolyte as well as adding organic solvents or modifiers (e.g. micelles, cyclodextrines, neutral polymers)[44]. Furthermore, because the surface of the fused-silica capillary is chemically reactive, it can be altered using chemical modification or dynamic coating to better control, minimize or even reverse surface charge[48]. Coated capillaries are useful for analysis of macromolecules, particularly proteins, which bind readily to the negatively charged surface of uncoated fused-silica capillaries[49,50].

1.2.2 CE instrumentation

The ability of CE method to perform a number of different types of separation derives from the simplicity and speed with which basic system elements or parameters can be varied. For the simplest part, this implies the altering of the background electrolyte composition by addition of various types of surfactants in MEKC, ampholites in CIEF or using the sieving matrix for CGE. Though all the above modes differ significantly in principle of separation, they can be readily performed on the same instrumentation with only minor modification in case of specific applications.

On the whole, the CE system is relatively simple and consists of vials containing a buffer and a sample, a capillary, two electrodes connected to the high voltage power supply, a detector and a data handling device. To perform electrophoretic separation, the capillary is filled with an appropriate background electrolyte and dipped into separate buffer vials. In most cases, a defined sample volume is introduced either electrokinetically or hydrodynamically by temporarily placing the capillary inlet into the sample vial. If the high voltage is applied to the system, the analytes start to migrate according to their mobilities. The separated zones passed through the detector are plotted as peaks on the electropherogram, where the migration time is indicated on the *x*-axis and the signal intensity is on the *y*-axis[1,44]. The diversity of available CE instruments is primarily dependent on the detector systems exploited, types of capillaries used, sample pre- or/and postmanipulations offered and the multiplicity of software for automation of sampling and data collection.

As follows from the method name, the capillary is a key element of the CE instrument. Advanced properties of the capillary material include chemical and electrical inertness, flexibility and robustness, as well as inexpensiveness. The characteristics of the fused-silica capillary satisfy most of these requirements and its thoroughly studied properties permit alteration of the EOF in the desirable manner. Various polymeric materials, such as a poly(methyl

methacrylate) (PMMA)[51,52], polyether ether ketone (PEEK)[51,53] and polytetrafluoroethylene (PTFE)[46,54] were also tested as a potential capillary material owing to the inertness to a wide range of organic and inorganic chemicals, stability at extreme pH and different surface chemistry. However, the demonstrated applications were limited by the mechanical softness and lack of the optical transparency of polymers[54,55]. Because most organic polymeric capillaries contain UV-absorbing functional groups, they are hardly transparent to the UV light below 270 nm. Thus, the complicated construction of the detection cell was needed certainly for photometric detection[52].

Detection in CE is commonly carried out using optical means (absorption and fluorescence)[56-58], which are standard on most commercially available systems. Alternatively, electrochemical detectors (conductometric, potentiometric and amperometric)[57,59,60] are inherently simpler in construction, easy to miniaturize and less expensive than optical devices, but have been still exploited comparatively little. New attractive features could be found by the use of the contactless conductivity detector in CE, which provides a universal detection with adequate sensitivity for most applications and enables integration into CE-based portable instrument[61,62]. On the other hand, the coupling of CE with a mass spectrometer results in a powerful system for the separation and structural characterization of a wide range of compounds. In spite of the low sample capacity in CE, the high sensitivity of modern MS instruments makes it possible to detect minor species in samples. Unfortunately, the online CE-MS combination presents several technical challenges, namely, the placement of the CE electrode at the MS interface, and solvent compatibility. Alternatively, the application of CE on-line sample preconcentration methods, as stacking, sweeping and isotachophoresis, could be used individually or in combination in order to enhance detection sensitivity[63].

The simplicity of instrumental design allows constructing home-made systems meeting special requirements for experimental conditions. Currently, research laboratories are the main leaders in CE instrument development using a wide variety of in-house manufactured systems and software. Most of these offer the versatility of sampling system integration, variety of detection modes and flexibility in coupling to other dimensions, as well as availability for postseparation sample treatment or fraction collection. Compared to home-made systems, commercially available instruments can be elaborated with enhanced facilities such as computer guidance of all operations, multiple injection devices and temperature control. From a practical point of view, automation of assays is the barest necessity for precise quantitative analysis in both scientific laboratories and diagnostic centres dealing with routine analyses. Nowadays the advanced CE instrument development is directed to the integration of separation in microfluidic devices and improvement of multidimensional strategies that enable analysis of compounds on the basis of more than one property.

1.2.3 CE of proteins – challenges and achievements in the field

The increasing number of various strategies and recent developments of electrophoretic modes opens new opportunities for the CE application to the high- throughput analysis and characterization of proteins. However, compared to the analysis of small charged compounds, protein analysis by CE has special features and is faced with unique challenges. Above all, proteins are polyelectrolytes and zwitterions whose net charge is contributed by the free α-amino group of the N-terminal residue, the free α-carboxyl group of the C-terminal residue and R-groups capable of ionization (Figure 2)[1,3]. Since the pI values of most proteins are in the pH range of 3 to 10, they are mainly positively charged at pH 3 or below and negatively charged at pH 10 or above.

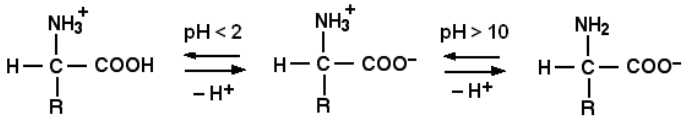

Figure 2. The effect of pH on the ionization of amino acids: a positive charge at a low pH, a zwitterion at a neutral pH and a negative charge at a high pH.

On the whole, the resolution and efficiency of protein and peptide separations can be varied simply by adjusting the pH of the separation medium. If CZE is performed in the fused-silica capillary, the low pH is more preferable because essentially all proteins are positively charged and migrate towards the cathode under the reduced EOF. In comparison, using higher pH buffers contributes to the strong protein adsorption onto the capillary wall. Generally, protein interactions with the charged fused-silica surface are the main limitation on the protein analysis by CE, which are caused by the presence of numerous charges and hydrophobic moieties on protein molecules. The adsorption of proteins severely degrades capillary electrophoretic performance appeared as a peak broadening, low separation efficiency, loss of resolution, poor reproducibility of peak areas and migration times[49,64].

In order to avoid these negative effects and to achieve high efficient and repeatable separation the undesirable interactions must be suppressed and the EOF controlled. Thereby capillaries with dynamic or permanent coatings have been developed over the years for a more precise analysis. Among the numerous coating materials presented, polymers, including poly(vinyl alcohol), poly(ethylene oxide), polyacrylamides and polybrene, exhibited the best performance[48-50,65,66]. Furthermore, an attractive strategy is using surfactants as wall coatings or buffer additives[50,67]. The applications of the modified silica surface to minimize the adsorption of proteins was demonstrated on numerous analyses of test mixtures and complicated real samples (tryptic digests, human body fluids, foodstuffs)[68-70]. Nevertheless, both the time-consuming and

complicated multistep derivatization procedure and the high cost of commercially available coated capillaries cannot be satisfactory for routine high-throughput analyses. Thus, while the most comprehensive separation has been achieved with coated capillaries, other approaches to minimizing protein adsorption propose the use of the extreme pH of BGE solutions, high salt concentrations, buffer additives and the proper conditioning of the capillary between the runs[51,67]. The improved results could be achieved by combining some strategies.

The main CE approaches that are presently applied to protein analysis include capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), micellar electrokinetic chromatography (MEKC) and capillary isoelectric focusing (CIEF)[17,18,71]. These modes exploit for separation various protein properties like charge, molecular weight, hydrophobicity and isoelectric point (Table 1).

Table 1. The commonly used CE modes for protein analysis with explanations of their separation principles

CE mode		Principle	Separation medium
Capillary Zone Electrophoresis	CZE	Charge-to-size ratio	Background electrolyte with appropriate pH and ionic strength
Micellar Electrokinetic Chromatography	MEKC	Charge-to-size ratio and hydrophobicity	Background electrolyte with surfactant micelles
Capillary Isoelectric Focusing	CIEF	Isoelectric point	Ampholyte mixture developing pH gradient
Capillary Gel Electrophoresis	CGE	Size (MW) and charge	Cross-linked and linear gels

Among the proposed modes of capillary electrophoresis, CZE is coupled with mass spectrometry most conveniently. Thus, the electrophoretically separated proteins can be identified using various mass spectroscopic techniques[21,71]. In addition to diverse range of applications in analytical scale using CE as a preparative approach to sample simplification or cleanup is also offered[72,73]. In that case, the off-line CE–MS is considered as a reasonable approach owing to its flexibility towards the optimization of both systems, possibility of additional intermediate manipulations and opportunity to use different types of ion sources for MS. The coupling of CE to MALDI is a promising tool for the protein analysis that favoured the development of new automatic fraction collection systems. The interfaces demonstrated have mainly differed in the way the CE effluent deposition on the MALDI plate, although the sheath-flow fraction collection set-up has proven to be the simplest[74-76]. In that case, a droplet of the CE effluent is mixed with the matrix solution at the capillary tip and can be

deposited directly on the target or collected into the individual vials with the subsequent treatment of fractions by chemical or biological means prior to transference into the mass spectrometer. The approach has been successfully applied to the analysis of model proteins[68,77,78] and real samples[79-81]. Also, multidimensional separation platforms have been used in combination with ESI-MS for the analysis of intact proteins[82,83]. Recently, fraction collection systems for the microchip CE have been demonstrated [73,84].

Besides the separation and quantification of complicated samples, biochemists are interested in parameters that cannot be estimated by most classical assays. The individual biochemical applications can be certainly performed by capillary electrophoresis owing to the method versatility. CE is ideally suited for enzyme-related analysis and has been widely utilized for the determination of enzyme activity and kinetics, as well as substrate and inhibitor screening[85-88]. Moreover, specially designed samplers approved on-line monitoring of the enzymatic conversion without disturbing the reaction progress and inconvenient manual procedures[89,90]. Striving for a further downscaling of the enzyme assay allowed integrate inside the capillary all the numerous operations of the enzymatic process from the mixing of reagents up to the final quantification of the reaction products. This electrophoretically mediated microanalysis (EMMA) proved to be highly valuable, particularly owing to the nanoliter consumption of enzyme and substrate, which is an essential characteristic in case of limited sample amount[40,91,92].

Much research was undertaken in the field of characterization of specific noncovalent protein interactions with other biomolecules with the aid of CE[43,93]. During the last decade, affinity capillary electrophoresis (ACE) has been successfully used to determine the binding constants for formed complexes, including protein-ligands, protein-metals, protein-peptides and protein-drugs[94,95,96]. The appearance of CE in the format of frontal analysis (CE-FA) facilitated the use of capillary electrophoresis for investigating of binding processes[97,98,99]. Guided by the separation principles of capillary zone electrophoresis and using ordinary commercial CE instrumentation, this approach affords an accurate determination of binding parameters. The key advantages of these techniques are the near-physiological conditions and rapidity of analysis, small sample volumes, ease of automatization and robustness, which are all extremely useful for the accurate and rapid characterization of binding processes.

2 EXPERIMENTAL

2.1 Instrumentation

2.1.1 Capillary electrophoresis

CE experiments were carried out using both a commercial instrument and specially designed home-made systems employing different types of capillaries and various detection modes.

The main part of electrophoretic measurements was made using in-house constructed electrophoretic equipments, which meet special requirements for application. These instruments were equipped with a high-voltage power supply (Spellman High-Voltage Electronics, Plainview, NY) providing the voltage of up to 20 kV. The security system cuts off the high voltage power supply if the box is opened. The experiments with home-made systems were performed at ambient temperature without temperature control. The samples were introduced either hydrodynamically (Δh) or electrokinetically using various injection times.

A bare fused-silica (Polymicro Technologies, USA) and polyether ether ketone (Upchurch, Oak Harbor, WA, USA) capillaries with an internal diameter of 75 μm and various total lengths were employed. The mixture of model proteins was separated in a poly(vinyl alcohol) coated silica capillary (Agilent Technologies, Waldbronn, Germany) of 52 cm (39 cm to the detector) in length and 50 μm i.d. × 365 μm o.d. dimensions.

The contactless conductivity detection was performed with a *TraceDec*® C^4D (Innovative Sensor Technologies, Strasshof, Austria) working with the *TraceDec Monitor* software. The CE platform for fraction collection was equipped with a UV absorbance detector using 210 nm for measurements. The detector signal was transferred to a personal computer via a self-made 16-bit analogue-to-digital-converter in a LabView (National Instruments, Austin, TX, USA) environment. All the data obtained were further processed by MATLAB Version 6.0 Release 12 (MathWorks Inc., Natick, MA, USA).

The CE-FA experiments were performed on a commercial Agilent CE apparatus (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array UV/Vis detector. The 50 μm i.d. fused silica capillaries (Agilent Technologies) were used and the temperature of the capillary in the cartridge was maintained by a thermostatic system. Prior to analysis the samples were thermostated at an appropriate temperature using a Techne DB-3A heater (Bibby Scientific Ltd, UK), and then were automatically injected using a hydrodynamic mode (pressure 50 mbar) at the anode. The ChemStation software (Agilent Technologies) was used for instrument control, data acquisition and data handling.

The pH was measured by using a 744 pH Meter (Metrohm, Herisau, Switzerland). A Milli-Q water purification system (Millipore S.A., Molsheim, France) (Millipore, Milford, USA) was used for producing the deionized water of high purity (18.2 MΩ/cm).

2.1.2 Fraction collection platform on the basis of CE

The design of the fraction collector is schematically presented in Figure 3. The cathode end of the capillary was housed inside a stainless steel needle using a coaxial liquid-sheath-flow configuration. A vessel with the sheath liquid was elevated relative to the stainless steel needle endpoint by approximately 10 cm providing a movement of the liquid by gravity with a flow rate of 2.5 µl/s. The resolved analytes in the CE capillary were sequentially fractionated into droplets (12 µl) that were collected into individual 250 µl PCR tubes located in the wells on a moving *xy*-stage on a microtiter plate. The CE separation and fractionation were completed in less than 10 min.

Compared to similar systems for fraction collection a new element in design was a droplet counter. The droplets were counted by a light detector with a built-in light emitting diode (LED) driver circuit (Optoswitch S4282-51, Hamamatsu). This only responds to the luminous flux of the connected LED, because the flux to the LED is modulated and a signal processor synchronizes the sensitivity of the light detector to the modulation. The unit can tolerate high background light levels. The output level is TTL/CMOS-compatible and readable using a parallel computer interface.

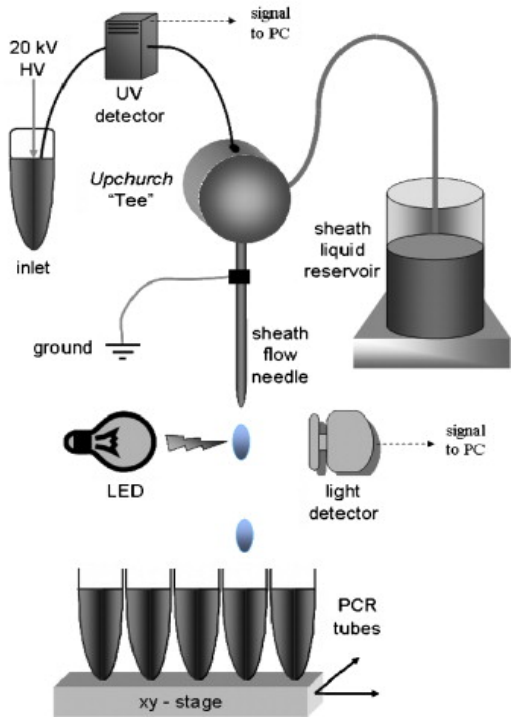

Figure 3. A schematic of the fraction collection system

2.1.3 MALDI-TOF

An Autoflex III MALDI-TOF mass spectrometer (Brucker Daltonics, MA,USA) equipped with a nitrogen laser (337 nm) and operated in the linear mode applying the accelerating voltage of 20 kV was used. The laser power was kept constant, and the other parameters were adapted automatically by the instrument's software. The spectrum were obtained by accumulating 500 laser shots in the positive mode of operation. Sample preparations were performed on a stainless steel plate using a dried-droplet technique.

2.2 Reagents

All chemicals were obtained at an analytical reagent grade and were used without additional purification.

Proteins - trypsinogen, lysozyme, cytochrome c, myoglobin, β-lactoglobulin (A+B), human hemoglobin and albumin from human serum (HSA, essentially fatty acid free) were purchased from Sigma-Aldrich (Steinheim, Germany), while ribonuclease A and α-lactalbumin were obtained from Fluka (Buchs, Switzerland). The HPLC peptide standard mixture was received from Fluka. Dicarboxylic acids (malonic, succinic, glutaric, phthalic, adipic and pimelic), rutin hydrate, quercitrin hydrate, flavone, warfarin, ibuprofen were also supplied by Sigma-Aldrich. Mesityl oxide used as an EOF marker was from Fluka.

The components of background electrolytes - 2-(*N*-morpholino)ethanesulfonic acid (MES), L-histidine (His), benzoic acid, acetic acid and sodium dihydrogen phosphate dehydrates were supplied by both Sigma-Aldrich and Fluka. The other reagents and solvents used in this work, as sodium hydroxide (NaOH), sodium dodecyl sulfate (SDS), sinapic acid, trifluoroacetic acid (TFA) and acetonitrile, were obtained from Sigma-Aldrich. Methanol was purchased from Rathburn Chemicals Ltd (Walkerburn, Scotland).

2.3 Material and methods

2.3.1 Preparation of solutions

The stock solutions of analytes at various concentrations were prepared from the corresponding compounds in the Milli-Q water and stored in the refrigerator. Except the working solution of human serum albumin prepared in the phosphate buffer for CE-FA measurements, proteins were also dissolved in the deionized water, stored at –20 °C and adjusted to room temperature prior to use. The stock solutions of flavonoids, warfarin and ibuprofen were dissolved in methanol and stored at +4 °C in the dark. All multicomponent solutions and mixtures were daily prepared from the corresponding stock solutions by dissolving them in the separation buffer in order to avoid system peak appearance and to prevent disequilibrium changes in the reaction mixture during the electrophoretic analysis.

The MES/His solution at a concentration of 15 mM (pH 6.1), a 250 mM acetic acid (pH 3) and a 20 mM benzoic acid (pH 3.1) were used as background electrolytes for electrophoretic separations. To improve its solubility benzoic acid was heated in the water bath at 65 ºC for 10 minutes. Fresh buffer solutions were mixed daily and were used at their natural pH values. The phosphate buffer solution at a concentration 67 mM was prepared from the sodium dihydrogen phosphate dehydrate in Milli-Q water adjusting the pH to 7.4 by the addition of a 1M NaOH. Prior to use, all the electrolytes and rinsing solutions were filtered through 0.45 μm nitrocellulose Millipore filters.

2.3.2 Capillary conditioning

Each new capillary was activated by rinsing with a 1 M sodium hydroxide solution for 20 min, then with a 0.1 M sodium hydroxide for 20 min and finally with the Milli-Q water for 20 min. For conditioning, at the beginning of each day and between the runs the capillary was flushed with a 0.1 M NaOH, the deionized water and the separation buffer for 3 min for each solution. In order to improve the reproducibility of CE frontal analysis the capillary was additionally washed with a 14 mg/ml SDS solution. This standard procedure was used for all fused silica and PEEK capillaries. The PVA capillary was preconditioned with a 10 mM phosphoric acid, the Milli-Q water and the buffer solution for three minutes each.

2.3.3 Procedures

In the fraction collection study a mixture of proteins (10 μM of each) was injected electrokinetically (at +20 kV for 8 s); the high voltage of +20 kV was applied. The 250 mM acetic acid was used both as the separation electrolyte and sheath liquid in order to match the ionic strength. Each collected fraction (12 μl) and matrix solution (2 μl) were mixed and briefly vortexed. Then 1.5 μl of the solution was spotted directly into the MALDI plate and allowed to air-dry at room temperature. A 10mg/ml solution of sinapic acid in a 0.1% (v/v) TFA/50% (v/v) acetonitrile/water was used as the matrix for the mass-spectral analysis of proteins. In order to improve the sensitivity of detection the procedure of sample deposition into the MALDI plate was repeated three times: the second sample layer was deposited on the top of the first layer, allowed to dry and then the procedure was repeated again.

For CE-FA analysis, a series of mixtures were prepared where the HSA concentration was kept constant (40 μM) and rutin, quercitrin or flavone solutions were added in different amounts to reach concentrations in the range of from 50 to 500 μM. The samples were thoroughly mixed and equilibrated for 30 min at 36.5 or 25 ºC. During the electrophoretic experiments the capillary was thermostated at an appropriate temperature as well. The hydrodynamic injection at 50 mbar at the anodic end of the capillary was used to introduce the sample and the high voltage of +8 kV was applied for separation. For displacement experiments the flavonoid and HSA solutions were thoroughly

mixed at a 1:1 ratio and then the site marker was added to the mixture at a concentration of 100 μM. Each flavonoid-HSA mixture was prepared and measured at least three times to ensure the reproducibility of the results.

2.3.4 Data processing and calculations in CE-FA experiments

The flavonoid-HSA binding as well as any ligand-protein interaction is described as an equilibrium process and can thus be mathematically expressed by the association constant (K_a):

$$[F] + [P] \leftrightarrow [FP]$$

$$K_a = \frac{[FP]}{[F][P]} \tag{4}$$

where F is the flavonoid (ligand), P is the protein and FP is the formed complex, whose binding constant is equal to K_a.

Assuming that the number of the same and independent binding sites in the protein is n, suppose that the equivalent concentration of the protein is $n[P]$ and the equivalent concentration of the complex is $n[FP]$. The indexes "t", "f" and "b" mean total, free and bound species in the solution. Then:

$$K = \frac{n[FP]}{[F]_f \; n[P]_f} \tag{5}$$

In that case $[P]_t = [P]_f + [FP]$ and $[F]_t = [F]_f + n[FP]$. Following the calculations, the amount of the bound flavonoid $n[FP]$ (further denoted by $[F]_b$) can be determined as the difference between the total $[F]_t$ and free $[F]_f$ flavonoid concentration:

$$[F]_b = [F]_t - [F]_f \tag{6}$$

For this purpose, a series of diluted solutions of rutin, quercitrin and flavone in the working concentration range were prepared and analyzed under the same conditions as were the HSA-flavonoid mixtures. The concentration of the flavonoid, which remained unbound after incubation with HSA, was found experimentally by measuring the height of the corresponding plateau peak on the electropherogram.

Further, substitutions of $n[FP] = [F]_b$ and $[P]_f = [P]_t - [FP] = [P]_t - [F]_b/n$ in Equation (5) and rearrangements will result in Equation (7):

$$K = \frac{[F]_b}{[F]_f \; (n[P]_t - [F]_b)}$$

$$[F]_b = K[F]_f \, n[P]_t - K[F]_f [F]_b$$

$$K[F]_f \, n[P]_t = [F]_b \left(1 + K[F]_f\right)$$

$$r = \frac{[F]_b}{[P]_t} = \frac{nK[F]_f}{1 + K[F]_f} \quad (7)$$

The binding curves for each flavonoid-HSA system were constructed by plotting the number of bound ligand molecules per protein molecule (r) against the free flavonoid concentration. Finally, the binding constant (K) and the number of sites (n) were determined by adjusting the data obtained to Equation 7 using a nonlinear regression. The *KyPlot* software package (version 2.0 beta 15; KyPlot, Tokyo, Japan) was used for this purpose, while the corresponding values were verified by the Solver algorithm.

The binding percentage of flavonoids to HSA was evaluated using the equation below:

$$B(\%) = \frac{[F]_b}{[F]_t} 100\% \quad (8)$$

The thermodynamic parameters dependent on temperature were calculated according to the following equations:

$$\ln \frac{K_2}{K_1} = \left(\frac{1}{T_1} - \frac{1}{T_2} \right) \frac{\Delta H}{R} \quad (9)$$

$$\Delta G = -RT \ln K = \Delta H - T\Delta S \quad (10)$$

where K_1 and K_2 are the binding constants at the corresponding temperatures T_1 and T_2, R is the gas constant, (ΔH) is the enthalpy, (ΔG) is the free energy and (ΔS) is the entropy changes on binding.

The logP values were estimated using the *miLogP* software version 2.2. (Molinspiration Chemoinformatics, Slovakia)[100].

3 RESULTS AND DISCUSSION

3.1 CE-C^4D with the polyether ether ketone capillary

Various organic polymers, like poly(tetrafluoroethene) (PTFE)[46,54], poly(methyl methacrylate) (PMMA)[51,52], ethylene vinyl acetate (EVA)[47], have been studied as materials for CE capillaries since they exhibit different surface properties. Schneider *et al.* used polyether ether ketone and PMMA capillaries with an inserted detection window made of a fused-silica capillary for photometric detection, but the complicated construction of the detection cell had an unfavourable influence on peak efficiency[52]. In the present study, the potential of the PEEK capillary for the CE separation was examined. The application of the contactless conductivity detector eliminated any need for the detection window and is suitable for the detection of optically inactive compounds. This promising combination of CE–C^4D with the PEEK capillary was employed for the development of a simplified protocol for the analysis of proteins, peptides and other analytes of biological origin. In order to verify the utility of polymer columns, the results were compared with those obtained using bare fused-silica capillaries. An attempt was made to demonstrate the performance of CE–C^4D with PEEK capillaries to be more favourable for distinct applications because of simple experimental conditions without any modification of the capillary surface or use of a complicated buffer composition.

3.1.1 Characterization of the PEEK capillary

To characterize the properties of the capillary surface, the direction and magnitude of the electroosmotic flow are used as principal parameters. Polyether ether ketone polymer is composed of repeating monomers of ketone and two ether groups. Unlike fused-silica, the PEEK material is inherently neutral and possesses no charged surface groups, considering a chemical structure (Figure 4)[55]. Nevertheless, experimental results demonstrated the presence of a relatively slow electroosmotic flow. Accordingly, a negative surface charge was supposed to exist in case of the PEEK capillary due to a cathodic direction of the EOF. The observed electroosmosis could be expected from the adsorption of buffer ions into the capillary surface or/and the presence of trace compounds in the column material[46,47].

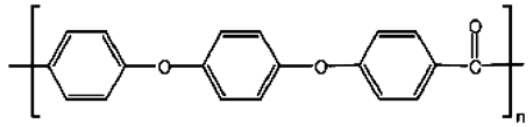

Figure 4. The chemical structure of polyether ether ketone polymer

The magnitudes of the EOF in the PEEK and fused-silica capillaries were compared by estimating the electrophoretic mobility of the neutral marker under the same experimental conditions. Using mesityloxide, the respective mobility was 2.65±0.04 $\times 10^{-8}$ m^2/Vs for the PEEK capillary (i.d. 75 μm; 7.5 mM MES/His buffer, pH 6.1). The value obtained well coincided with literature data 2.6×10^{-8} m^2/Vs (10 mM MES/His buffer, pH 6)[53]. In comparison, the EOF in the fused-silica capillary was considerably stronger, being 6.42×10^{-8} m^2/Vs. At the same time, the stability of the EOF on the PEEK column was examined. This was found to be highly reproducible relating to the run-to-run RSD for the EOF mobility of 1.4% (n=8).

In the present study, using PEEK capillaries in combination with the contactless conductivity detector allowed analysis without any need for the detection window. Thus, the lack of optical transparency was no hindrance henceforward. The only noted drawback was the mechanical softness of the PEEK material which caused some technical problems, particularly during the installation of the capillary into the detection cell.

3.1.2 Fields of application

Peptides and proteins are one of the biological compounds whose analysis is the most essential but difficult to accomplish by CE. In practice, the manipulation of the pH of the buffer is the simplest tool used to vary the charge of a compound and, hence, to control the resolution between the analytes in the mixture. At high pH, peptides and proteins are mainly negatively charged and, furthermore, the possible adsorption will be minimized as a result of the charge repulsion effect. The 20 mM Tris (pH=8.9) and 100 mM ammoniun hydroxide (pH=9.8) were tested as separation buffers due to their compatibility with the C^4D detector. However, based on poor experimental results, it was assumed that the EOF in the PEEK capillary is too weak to enable the negatively charged high-molecular analytes to be pushed to the cathode direction. Further, the possibility of separation performance in the PEEK capillary at low pH was examined. Then the pH of the buffer is well below pI, all the proteins and peptides readily migrate to the cathode having a positive charge. After a series of experiments, the 20 mM benzoic acid as a background electrolyte (pH 3.1) and the separation voltage of +19 kV with electrokinetic injection proved to be optimal conditions for peptide and protein separation.

First, the analysis of a peptide mixture (Gly-Tyr, Val-Tyr-Val, Met-enkephalin, Leu-enkephalin, angiotensin II) was carried out in the PEEK capillary using the experimental conditions found. Figure 5A illustrates the successful CE separation of peptides within 12 minutes according to an increase in their molecular weight. A minimum resolution was achieved between Met-enkephalin and Leu-enkephalin, which differ in only one amino acid residue. The electropherogram of the standard peptide mixture obtained in the fused-silica capillary at the same conditions is also shown in Figure 5B. The analysis of all compounds was achieved even in a shorter time. Furthermore, the

electropherograms observed show that the two separation patterns are markedly different. Moreover, even peptides without one of the three amino acids absorbing the UV light (Phe, Trp, Tyr) can now be directly measured by the C^4D detector.

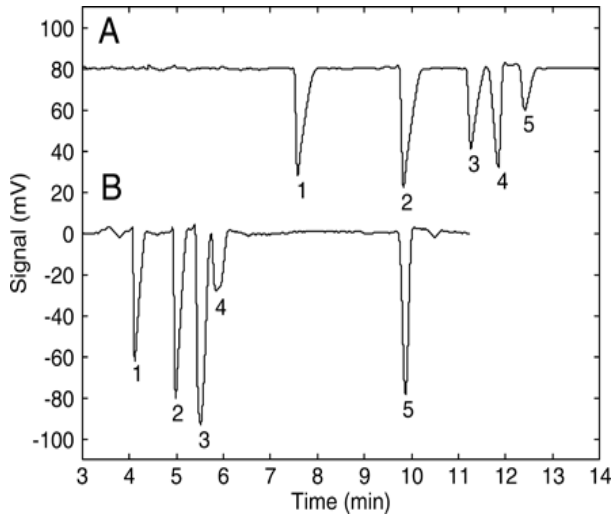

Figure 5. The electrophoretic separation of the peptides mixture in (A) PEEK and (B) fused-silica capillaries. Experimental conditions: capillary 75 μm i.d. × 64 cm × 44 cm, 20 mM benzoic acid (pH 3.1), separation voltage +19 kV, electrokinetic injection 5 s at +19 kV, concentration of each peptide 0.25 mg/mL. Peak identification: 1 - Gly-Tyr, 2 - Val-Tyr-Val, 3 - Met-enkephalin, 4 - Leu-enkephalin, 5 - angiotensin II.

Then, the analysis of a model mixture consisting of anionic and cationic proteins (cytochrome c, myoglobin, ribonuclease A, β-lactoglobulin A+B, α-lactalbumin) with different pIs (4.5-10.6) and molecular weights (12.4-24 kDa) was carried out in a single run without any modification of the buffer composition or capillary wall. The electropherogram of the resolution achieved between all the tested proteins in the PEEK capillary is presented in Figure 6. The analysis was fast (around 9 min) and the results were obtained at a concentration of proteins of 0.5 mg/mL. As the pH of the buffer is well below the pI values of basic proteins, the latter are migrated prior to acidic proteins because of the higher positive charge. The results obtained using the fused-silica capillary at the same experimental conditions were unsatisfactory and, therefore, are not shown.

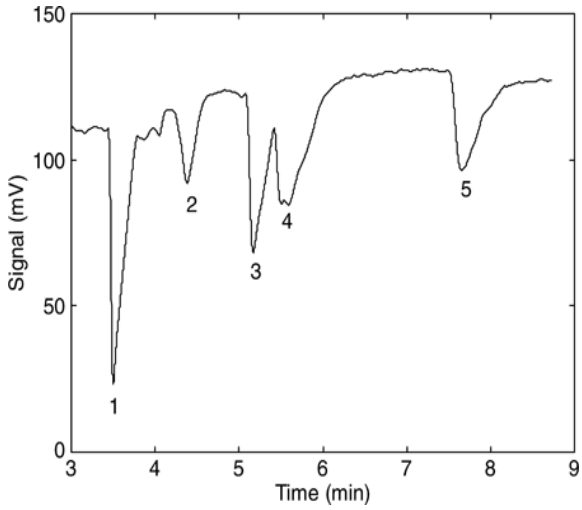

Figure 6. The electrophoretic separation of proteins in the PEEK capillary. Experimental conditions: PEEK capillary 75 μm i.d. × 55 cm× 30 cm, 20 mM benzoic acid buffer pH 3.1, separation voltage +19 kV, electrokinetic injection 10 s at +19 kV, concentration of proteins in the sample 0.5 mg/mL. Peak identification: 1 - cytochrome c, 2 - myoglobin, 3 - ribonuclease A, 4 - β-lactoglobulin A/B, 5 - *α*-lactalbumin.

As demonstrated above, the naturally low electroosmotic flow in the PEEK capillary had a limitation in the analysis of high-molecular biomolecules at high pH. However, this special feature could potentially have an advantage in the separation of small ions having more than one negatively charged group, for example, dicarboxylic acids. Since the pK_{a1} of the acids is in the range of from 2.8 to 4.3 and the pK_{a2} of the second carboxyl group is from 5.4 to 5.7, both carboxyl groups are deprotonated at the neutral pH of the buffer. Owing to the high negative electrophoretic mobility of analytes the application of the reversed polarity with anodic detection is more preferable. As a rule, cationic surfactants are then used to reduce or even reverse the direction of the EOF and to achieve separation. Furthermore, as mentioned above, using the conductivity detection served well when the analytes of interest do not contain UV-active groups. This is the case with dicarboxylic acids which usually need previous derivatization or indirect UV detection. Using the combination of CE–C^4D with the PEEK capillary these complicated manipulations are no longer necessary.

The separation of the mixture of dicarboxylic acids (namely malonic, succinic, glutaric, phthalic, adipic and pimelic acids) was easily performed in the PEEK capillary without any prior surface coating (Figure 7A). The separation was rather fast as the experiment was accomplished in less than 8 min. The baseline resolution was achieved using a 15 mM MES/His buffer (pH 6.1), an applied voltage of +19 kV and sample electrokinetic injection from the cathodic side with a further anodic C^4D detection.

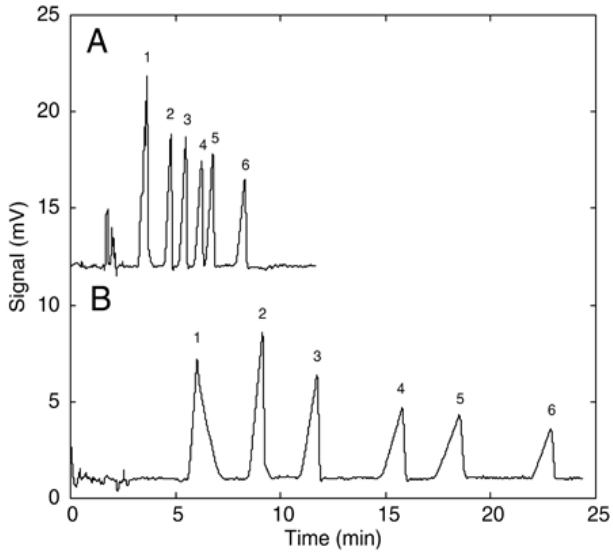

Figure 7. The separation of dicarboxylic acids in PEEK (A) and fused-silica (B) capillaries. Experimental conditions: capillary 75 μm i.d.×46 cm×26 cm, 15 mM MES/His buffer pH 6.1, separation voltage +19 kV, electrokinetic injection of 50 μM standards for 3 s at +19 kV from the cathodic side. Peak identification: 1 - malonic acid, 2 - succinic acid, 3 - glutaric acid, 4 - phthalic acid, 5 - adipic acid, 6 - pimelic acid

In comparison, the electropherogram of the separation of dicarboxylic acids in the fused-silica capillary performed at the same electrophoretic conditions is demonstrated in Figure 7B. As seen, the time of analysis is twice longer and the peak shapes are broadened. The explanation for this phenomenon could be the difference in EOF velocity between the capillaries. Having two negatively charged groups, dicarboxylic acids can easily overcome the slow EOF in the PEEK capillary, but the movement of sample zones in the fused-silica capillary suffers from the strong counteractive electroosmotic flow.

3.1.3 Reproducibility and efficiency

The performance of the PEEK capillary appeared to be different from that of the fused-silica, because of a polymeric nature of the surface. The ability of the polyether ether ketone material to limit protein adsorption was estimated by monitoring separation efficiencies and the repeatability of the migration time and peak areas. These important parameters were also calculated for the other compounds under study and are summarized in Table 2. On the whole, the results indicate that the PEEK capillaries demonstrated a moderate reproducibility and efficiency.

Table 2. The performance criteria of the C^{4}D-PEEK capillary system

Analyte	RSD (%)[a]		Efficiency (plate/m)[b]	LOD (μM)[c]
	Migration time	Peak area		
Cytochrome c	5.8	7.4	9084	9.0
Ribonuclease A	6.1	6.7	13 693	8.2
Myoglobin	6.8	7.0	11 907	8.6
α-Lactalbumin	7.3	10.3	7840	12.5
β-Lactoglobulin A/B	7.5	10.4	10 929	12.6
Gly-Tyr	5.5	3.6	20 236	11.4
Val-Tyr-Val	5.8	5.9	28 116	18.7
Met-enkephalin	5.5	4.9	45 182	15.4
Leu-enkephalin	6.0	3.3	75 427	10.3
Angiotensin II	5.5	3.1	55 995	9.7
Malonic acid	5.7	4.1	9013	6.2
Succinic acid	5.5	6.4	10 155	9.6
Glutaric acid	6.3	4.6	13 624	6.9
Phthalic acid	7.1	5.1	20 183	7.7
Adipic acid	7.7	3.9	15 783	5.9
Pimelic acid	7.4	4.7	18 410	7.1

a) The values were obtained as an average of at least of three independent runs
b) The number of theoretical plates was calculated as $N=5.54[t_i/(area_i/peak\ height_i)]^2$ and recalculated to the effective length of the capillary
c) The limits of detection were calculated as a triple ratio of the mean peak area to the standard deviation of areas

The highest separation efficiency was achieved in case of using the PEEK capillary for the peptide analysis. The number of theoretical plates obtained amounted to 75 000 plates/m. Furthermore, the RSD values of the migration time and peak area did not display any marked trend for extreme pH conditions, supposing the chemical stability of the PEEK capillary at various buffer systems. On the other hand, the calculated *N* values indicated a rather low effectiveness (7840-13 693 plates/m) of protein separation if the PEEK capillary was applied. Moreover, the relative standard deviations of the migration time and peak area were close to 7 and 10%, respectively.

The shifts in the migration time and low efficiency indicate that the adsorption of proteins onto the PEEK capillary wall was not completely avoided. Theoretically, the electrostatic interactions and adsorption-desorption processes can affect the band width of the protein zone as migration occurs. Especially at extreme pH, the hydrophobic areas of protein could be exposed, resulting in the hydrophobic adsorption. Taking into account the hydrophobicity of the PEEK material, an undesirable interaction between the capillary wall and protein may take place, leading to the broadened peaks. The variation of the electrolyte pH was evidently insufficient for efficient separation, and so strategies to control the adsorption of protein samples onto the polymer material needs to be further developed.

In the case of the dicarboxylic acids separation, the RSD of the migration time and peak area was estimated to be from 5.5 to 7.7 % and from 4.1 to 6.4 %, respectively. The movement against the EOF could explain the variation in migration time, broadening of the sample zone and distortion of the peak shape resulting in the decrease in the separation efficiency. The *N* values for the PEEK capillary are in the range of from 9013 to 20 183 plates/m. In comparison, the electroosmotic flow in the fused-silica capillary offers a more stronger resistance to the movement of sample zones leading to a three-fold increase in the analysis time and a dramatic reduction in the efficiency (*N* = 2824-13107 plates/m).

The limited reproducibility can also result from the precisely unclear nature of the EOF and the use of electrolytes with a low buffering capacity due to the exploitation of the conductivity detector. The proper selection of background electrolytes is really important for the success of any CE separation, but this choice is particularly critical in case of a combination of capillary electrophoresis with the C^4D detection. With the aim to obtain high separation efficiencies, matching the buffer ion mobility with analyte mobility is necessary for minimizing peak shape distortions. But, in order to maximize sensitivity, it is necessary to achieve the highest possible difference in mobility between the analyte ion and the BGE co-ion. As a consequence, finding a suitable background electrolyte is a compromise between separation efficiency and detection sensitivity, which is difficult to accomplish for CE-C^4D systems at the same time. Furthermore, the availability of BGEs with appropriate pH is especially limited in case of a need to affect a solute charge.

The quantitative response of CE-C^4D in the PEEK capillary to analytes was also investigated. The limits of detection were determined as concentrations giving the ratio of mean peak area (A_{mean}) to areas standard deviation (sA) equal to three (*i.e.* $S/N=3*A_{mean}/sA$). For non-stacking sample injection the LOD values are relatively high, being in the micromolar range. Sensitivity could be further increased in case of the method development for a definite application.

Overall, the results obtained proved that the untreated PEEK capillary is improper for a precise analysis of complex protein mixtures and may be used only as a preliminary express test or preparative step prior to identification. On

the other hand, PEEK capillaries are considered as an attractive alternative to the traditional fused-silica capillaries for distinct applications, especially for the analysis of small molecules as peptides, and are demonstrated to be a promising potential for developing portable, point-of-care devices for *in situ* analysis. Further applications of PEEK capillary to the analysis of macromolecules with a view to obtain accurate quantitative results are needed for preliminary consideration taking into account the hydrophobic nature of the polymeric material. A more precise analysis of individual analytes in real samples, which is complicated due to peak overlapping, as well as the identification of unknown species requires a particular approach considering the sensitivity and selectivity of mass spectrometric instruments. To reduce sample complexity prior to mass spectrometry, fraction collection is one of the effective approaches carried out prior to protein analysis.

3.2 Fraction collection system for the CE-MALDI-TOF-MS analysis of proteins

3.2.1 The design features of the CE fraction collection platform

The CE system presented for fraction collection provides improved performance due to the introduction of new aspects. In the present work, CE is completely autonomous and the collection of fractions in the form of droplets enables all problems related to the electrical coupling between CE and MS to be avoided. Unlike several other fraction collectors, no interruption of the separation voltage during the experiment is needed.

Furthermore, it is well known that the most widely used background electrolytes required for CE separation are not suitable if capillary electrophoresis is coupled to MS in an on-line mode. In case of the off-line mode this obstacle was easily overcome by using instead of an outlet buffer reservoir continuously following droplets of the sheath liquid, which is compatible with MS. Furthermore, the migration speed of sheath-liquid ions must be smaller than the velocity of the electroosmotic flow on the anionic side in order to prevent intervention with the separation process in the capillary. The experimental mobilities of the EOF (25 mM sodium tetraborate buffer, pH 9.3) and an acetic acid ion were 6.4×10^{-8} m^2/Vs and -2.6×10^{-8} m^2/Vs, respectively, meaning that sheath-liquid ions would probably not migrate into the CE capillary. Acetic acid was used both as a sheath liquid and background electrolyte in protein analysis for the purpose of preventing disturbance of the CE separation in the PVA capillary with the suppressed EOF and approval for MS experiments.

The time of falling of a droplet was precisely correlated with the migration time of the peak observed at the UV detector. Assuming that the mobility of the analyte is constant during electrophoresis, the time at which the analyte band reaches the capillary outlet (t_o) is calculated from the migration time corresponding to the top of the analyte peak as its zone passes through the detector window (t_d). The calculation is performed as follows: $t_o = (l_o/l_d)t_d$,

where l_o is the full length of the capillary and l_d is the effective length of the capillary to the detector. The negligible increase in time interval between the droplets falls-off could be observed if the sheath liquid is delivered by gravity owing to a gradual decrease of the level of the liquid in the vessel. However, the proposed solution in the form of a droplet counter allowed avoiding the presumable drawback.

3.2.2 Validation of the system with a model mixture of proteins

The proposed off-line coupling of CE to MALDI-TOF could certainly be useful for the analysis of complex proteomic samples due to the possibility of prior fractionation for simplification of MS profiles, as well as for removal of species which interfere with the MALDI analysis. The fraction collection system developed was evaluated on a model mixture of proteins consisting of cytochrome *c*, lysozyme, ribonuclease A, trypsinogen, α-lactalbumin, human hemoglobin and human serum albumin. As already demonstrated, the separation of proteins is not easily performed by CE. Reliable strategies use electrolyte solutions at extreme pH values and/or capillaries with a coated inner surface. On the assumption of this, protein separation was performed in the PVA coated silica capillary using a 250 mM acetic acid (pH 3) as a background electrolyte. The electropherogram of the above-mentioned mixture with good resolution is demonstrated in Figure 8A. Additionally, the droplet counter signal and numbering of fractions through one are shown at the bottom of the electropherogram. The time interval between two consecutive droplets falls-off corresponds to the time during which a droplet is formed.

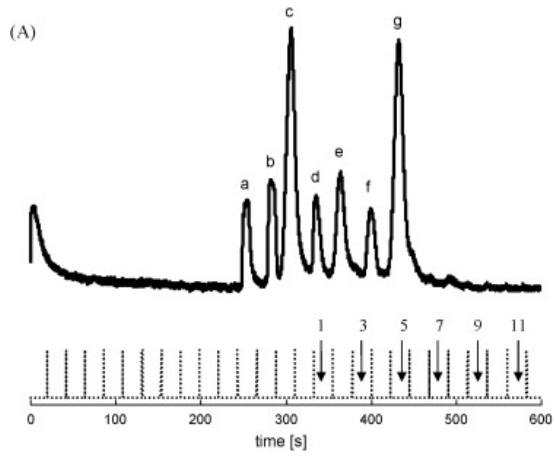

Figure 8. The CE-MALDI-TOF analysis of protein standards: (A) electropherogram with an electrical impulse, (B) mass spectra of the collected fractions. Experimental conditions: PVA capillary 50 μm i.d.×52 cm×39 cm, 250 mM acetic acid as a background electrolyte and sheath liquid, separation voltage +20 kV, electrokinetic injection 8 s at +20 kV, each protein concentration 10 μM, UV detection at 210 nm. Peak identification: a – cytochrome *c*, b – lysozyme, c – human serum albumin, d – ribonuclease A, e – trypsinogen, f – α-lactalbumin, g – human hemoglobin.

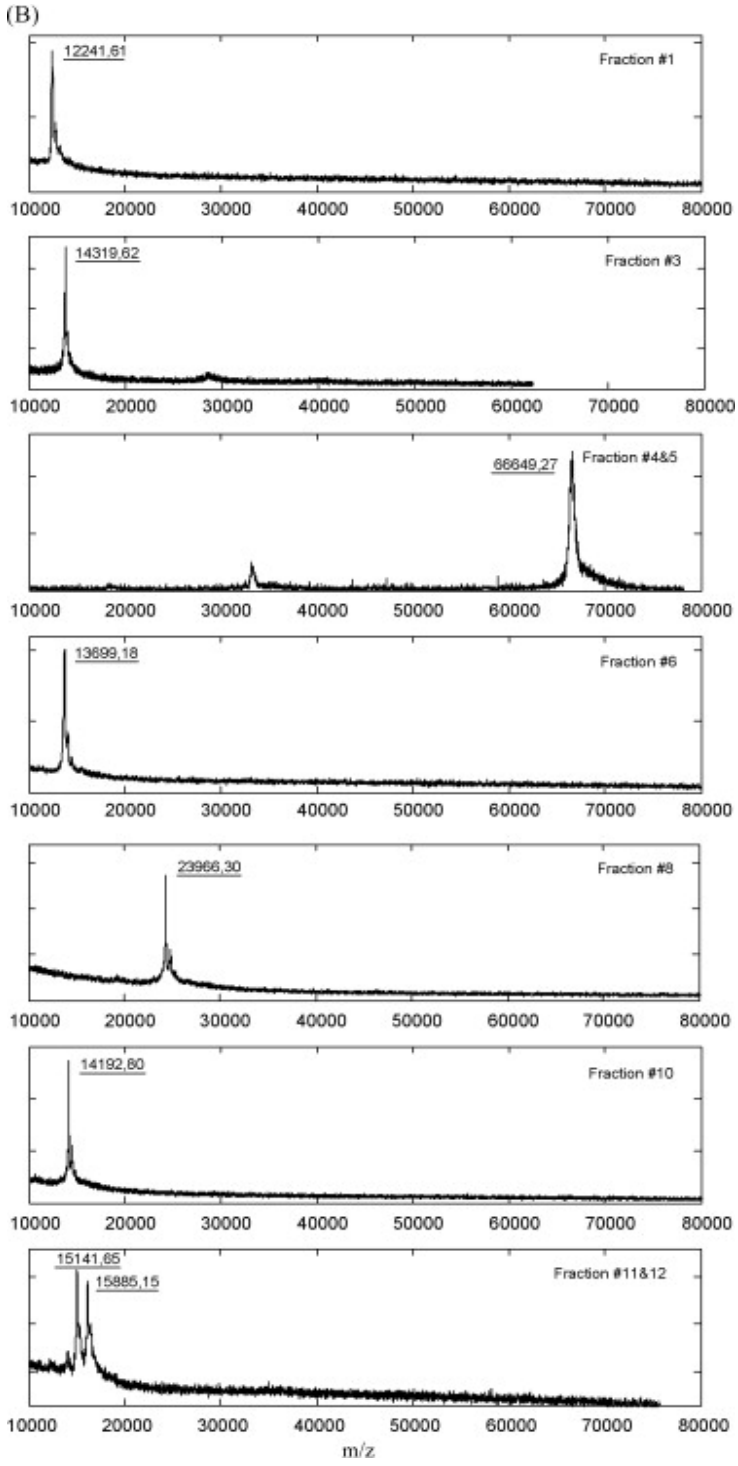

Figure 8. (Continued)

The procedure of sample collection into vials is simple and allows a variety of subsequent specific tests to be performed on individual fractions in order to gain the required chemical characteristics. Moreover, the size of the collected droplets is ergonomically compatible with human operator capabilities, which enables further manual manipulations of the fractions. In this case, mixing with the matrix solution and deposition on the stainless steel plate was performed for a MALDI-TOF analysis, but a variety of other possibilities (fraction concentrating, tryptic digestion, etc.) obviously exists. A further system development will allow collecting fractions by a robotic spotter, while the MS analysis is possible using an autosampler.

The corresponding mass spectra of the collected fractions are shown in Figure 8B, with the exception of those fractions that appeared to be empty or whose amount of protein was negligible. In the spectrum recorded, the main ion peaks $[M + H]^+$ were detected. In general, the major peaks observed in each mass spectrum correspond to the proteins expected according to the CE signal in Figure A. The mass spectrum of fraction #1 contains only a single peak of cytochrome *c*. In fraction #3 the signal from lysozyme is observed. The next two mass spectra are indicative of the presence of human serum albumin in fractions #4 and #5, respectively. A peak in the mass spectrum of fraction #6 is identified as ribonuclease A. In fraction #7 negligible quantities of ribonuclease A and trypsinogen are observed, but the main signal of trypsinogen is detected in the subsequent fraction #8. The mass spectrum of fraction #10 contains a peak of α-lactalbumin. Hemoglobin is identified in fractions #11 and #12 containing two neighbouring peaks. This fact could be explained by a tetrameric structure of hemoglobin, consisting of two α and two β subunits which are bound non-covalently and dissociate during the ionization step. A variation in signal intensity was also observed between different fractions: the mass spectrum of low molecular weight proteins results in a higher signal intensity probably due to the different ionization efficiency.

The data on the reproducibility of the electrophoretic separation of proteins with subsequent MALDI-TOF-MS measurements are presented in Table 3. The obtained RSD values of up to 3.5 % correspond to the prevented adsorption of proteins in CE runs owing to the high precision of the PVA coated capillary and guarantee an accurate distribution of analytes between the predicted fractions. The results of MALDI-TOF measurements also demonstrated an excellent stability, because from run to run the RSD of the experimental molecular weight was less than 0.3 %.

Table 3. Reproducibility statistics on CE-MALDI-TOF measurements

Protein	RSD (%)[a]		Molecular weight (Da)		
	Migration time	Peak area	Literature[b]	Experimental[a]	SD[a]
Cytochrome *c*	1.8	2.5	12300	12225.75	35.38
Lysozyme	2.3	3.0	14300	14301.60	44.07
Human serum albumin	1.6	3.3	66500	66616.99	157.85
Ribonuclease A	2.5	1.4	13700	13695.88	12.11
Trypsinogen	1.9	3.2	24000	23985.63	66.91
α-Lactalbumin	1.4	2.5	14200	14179.32	34.34
Human hemoglobin	1.9	3.5	64000	15142.14	15.23
				15884.89	12.15

a) RSD, SD and experimental average are based on 5 independent replicate measurements

b) An approximate molecular weight from the reagents producer's datasheets

The reduced sensitivity is a well-known drawback of using a CE instrument as a fraction collector because of sample dilution by the flow of an auxiliary liquid. A dilution factor estimated here is about three orders. This is partially compensated for by the high sensitivity of the MS detection. The 10 μM protein mixture introduced into the capillary for fraction collection was found to be optimal for the CE-MALDI-TOF-MS combination. The necessity of decreasing detection limits can lead to an increase in the amount of the injected sample, which predictably leads to the loss of resolution on the electropherogram. If the identification of a peak is an issue, the scarifying separation quality for increasing the detection limit could be certainly justified. On the other hand, in the proposed approach it can also be minimized by using a preconcentration step for collected fractions or repeating the deposition of the sample on the MALDI plate.

In general, the type of the fraction collection developed in the present work could be envisioned as supporting a project concerning the study of a high number of samples with similar composition, like the profiling of bioactive compounds and biomarker discovery, for example. Although current experiments demonstrated the use of the MALDI-TOF as a detector in the second dimension for protein analysis, the fraction collection system developed was applied to other stand-alone MS instruments as well. The proposed off-line approach was successfully used to investigate real plant extracts by a micro-column RP-HPLC-ESI-Q-TOF-MS and ICP-MS as reported in Paper II.

3.3 Study of protein interactions with bioactive compounds

In addition to the demonstrated applications of CE to protein analysis, capillary electrophoresis was proposed as an attractive technique for the investigation of the binding processes of protein with biologically important compounds. The application was focused on the characterization of interactions between human serum albumin and naturally occurring flavonoids, namely, flavone, quercitrin and rutin. Flavonoids have received meticulous attention due to the numerous evidences of their beneficial effects on human health, wide-ranging biological activities and potential use as natural drugs. Thus, the study can contribute to a better understanding of their transportation mechanism, as well as prediction of their physiological behaviour based on structural specificities.

The capillary electrophoresis frontal analysis (CE-FA) is one of the numerous modes of CE, which is used to characterize the protein interaction behaviour at near-physiological conditions. The enormous studies of interactions between ligands and biomolecules have been principally limited to the estimation of binding constants, but the present work attempted to demonstrate the possibility of carrying out a more comprehensive assay using the CE-FA technique.

3.3.1 Optimization of CE-FA conditions

The capillary electrophoresis frontal analysis is based on injecting a larger amount of sample into the capillary than needed for separation. As a result, the peaks of the analytes have a trapezoidal form with a definite plateau on the top. Therefore, the quantities of bound and free ligands needed for the construction of building curves and calculation of binding constants can be estimated using the corresponding heights of the plateau on the electropherograms. Furthermore, the CE-FA required a difference in electrophoretic mobility of the free ligand from the protein and the protein-ligand complex for a precise estimation of the plateau height. The compliance with these requirements was studied at first.

Under conditions close to physiological quercitrin and rutin are slightly negatively charged due to the presence of partly dissociated phenolic -OH groups with a pKa in the range of from 6.74 to 11.65[101]. Flavone, having a zero electrophoretic mobility, migrated with the electroosmotic flow under these conditions. Human serum albumin (pI=4.7) is mostly negatively charged in the physiological pH range and due to its high molecular mass HSA tended to migrate slowly towards the anode. Furthermore, it was suggested that the flavonoid-HSA complex and HSA migrated as a single zone because a bound flavonoid does not notably change the electrophoretic mobility of protein. On the electropherogram, flavonoid and the flavonoid-HSA complex (co-migrated with a free protein) were observed as completely separated ordinary CE peaks when the injection time was short. In order to investigate plateau formation, different injection times from 5 up to 60 s were examined (Figure 9).

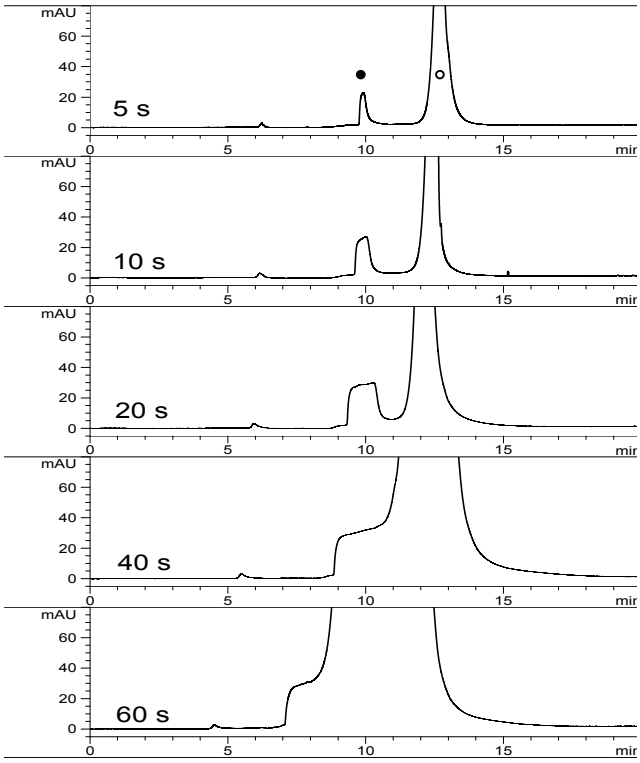

Figure 9. The effect of the injection time on plateau formation. Experimental conditions: 67 mM phosphate buffer (pH 7.4), capillary 50 μm i.d. × 37 cm × 45 cm, applied voltage +8 kV, hydrodynamic injection at 50 mbar, UV detection at 210 nm; sample: 150 μM rutin (•) and 40 μM HSA (○).

As seen in Figure 9, the plateau region of the free flavonoid started to appear with the 20 s injection, but a more evident plateau was observed when the sample injection time was about 40 s. Moreover, with increasing injection time, the peaks became broadened and equilibrium was maintained in the overlapping zone. Thus, the injection time of 40 s at a pressure of 50 mbar was chosen as an optimal injection time for all further experiments.

3.3.2 Estimation of binding constants of flavonoids to human serum albumin

The interactions between human serum albumin and flavone, rutin and quercitrin were studied according to the above-mentioned procedure under the optimized experimental parameters and at near-physiological conditions (67 mM phosphate buffer, pH 7.4; temperature at 36.5°C). The binding of a ligand to a protein can be described by an equilibrium process, when one part of the ligand is presented in the free form and the other part is in a complex with

protein. Typical CE-FA electropherograms on an example of a standard flavone and the flavone-HSA mixture are illustrated in Figure 10. As can be observed, the height of the plateau corresponding to the flavone decreased in the presence of human serum albumin, thereby proving the formation of the complex.

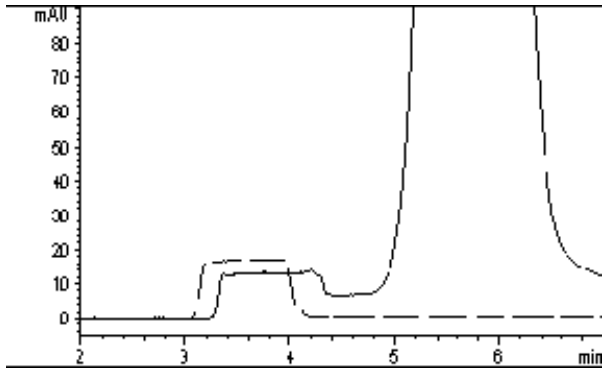

Figure 10. The electropherograms of flavone (---) and flavone/HSA mixture (—). Experimental conditions as in Figure 10, hydrodynamic injection at 50 mbar for 40 s; samples: 300 μM flavone and 300 μM flavone + 40 μM HSA.

By repeating the analysis at various protein/ligand ratios, the free ligand concentration was determined from the height of the unbound ligand plateau. The difference between the total and free ligand concentrations corresponds to the amount of the ligand present in the complex (Eq. 6). Following the calculation, the number of bound ligands per protein ($r = [F]_b/[P]_t$) was estimated for each protein-ligand system (Eq. 7). Finally, based on the experimental data and calculations performed, the binding curves were plotted as r parameter versus free flavonoid concentration as shown in Figure 11.

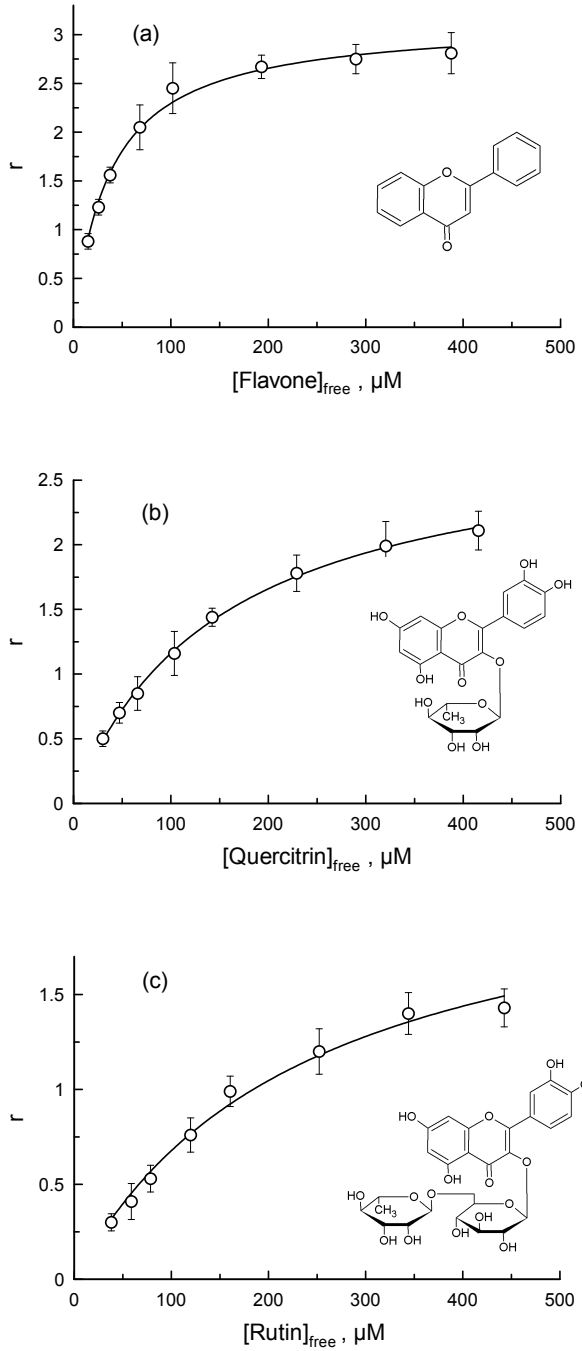

Figure 11. The binding curves of the flavonoids inverstigated: (a) flavone, (b) quercitrin and (c) rutin

Fitting the experimental data to the Equation 7 using nonlinear regression, the binding constants (K) and the number of binding sites per protein molecule with the same affinity (n) were determined for the investigated flavonoids and are summarized in Table 4. As follows from the results, the studied flavonoids exhibited moderate binding properties towards HSA with essential differences in the binding constants. Based on the obtained *K* values, the binding order could be presented as follows: flavone (21.39×10^3 M^{-1}) > quercitrin (6.64×10^3 M^{-1}) > rutin (4.13×10^3 M^{-1}).

Table 4. The binding parameters obtained by the CE-FA method (*36.5 °C*) and the lipophilicity (*logP*) values of the flavonoids under study

Compound	K ± SD ($\times10^3$ M^{-1})	n ± SD	*log P* [a)]
Flavone	21.39 ± 0.55	2.26 ± 0.02	3.737
Quercitrin	6.64 ± 0.10	2.91 ± 0.02	0.644
Rutin	4.13 ± 0.16	2.32 ± 0.04	-1.063

a) Calculated by the miLogP software version 2.2. available as freeware

The available data obtained using conventional methods were consistent with the results of the present study, despite the different measurement principles. The reported binding constants for rutin measured by the fluorescence spectroscopic technique varied from 4.1×10^3 M^{-1} up to 105×10^3 M^{-1} depending on the experimental conditions and the calculation procedure used[102,103]. The results showed better agreement when the same technique and similar conditions were used. For example, Lu *et al.* reported a binding constant for the rutin-HSA complex equal to 1.57×10^3 M^{-1} measured by the CE-FA method[104]. Diniz *et al.* presented the binding constants for rutin (6.9×10^3 M^{-1}) and flavone (33×10^3 M^{-1}) obtained by capillary electrophoresis, which were very close to the values presented in the current study[105].

Additionally, on the example of rutin, the binding capability to HSA was estimated by quenching the intrinsic fluorescence of the protein under similar experimental conditions as used with the CE-FA method. The calculated binding constant of rutin to HSA was $(6.4 \pm 0.8)\times10^3$ M^{-1}, which was well compatible with the results obtained by the CE-FA. This confirms the suitability of method for estimation of ligand-protein binding constants. Variations in results probably arise from differences in experimental conditions and selected methods, as well as from the data processing that follows.

3.3.3 Relationship between the structural specificities and the binding constants

The results obtained were discussed based on the structural specificities of the flavonoids under study (Figure 11). As shown in Table 4, flavone had the highest value of the binding constant, which presents the basic structure of flavonoids. In comparison, quercitrin and rutin contain monoglycoside rhamnose and diglycoside rutinose (rhamnose + glucose) at the C(3) position, respectively. This fact was reflected on the binding constant, whose value was about three times lower in the case of quercitrin and approximately five times lower in the case of rutin than that of flavone. The differences in affinity could be explained by an increased molecular size and, as a consequence, less flexible and bulky structures of glycosides. This could result in a less favourable placement within the binding site, so that the binding capacity of either quercitrin or rutin is lower than that of flavone. Besides, the additional polar groups of sugar moieties render these molecules less hydrophobic which could thus effect their orientation in relation to the hydrophobic environment of the HSA. Consequently, it was concluded that the presence of sugar moieties, as well as the number of saccharides, had a marked effect on the binding properties of flavonoids to human serum albumin. On the whole, the glycosylation of flavonoids decreased their affinities for protein.

Furthermore, Table 4 presents the *logP* values of flavone, quercitrin and rutin, whose magnitudes were predicted on the basis of individual group contributions to the total lipophilicity. Referred to as a measure of lipophilicity, the *logP* value is an important criterion for the prediction and understanding of drug behaviour in the human body in early pharmacological studies. It can be concluded from the data obtained that the lipophilicity (*logP*) of the tested flavonoids was intermediate and decreased after glycosylation. Moreover, the *logP* values of flavonoids were correlated well with their binding constant values. The more hydrophobic flavone (*logP* = 3.737) had a high binding constant and could be found in the plasma mainly bound to the HSA, improving its solubility and prolonging half-life. In addition, a marked decrease in lipophilic characteristics as well as in the binding properties was demonstrated by two glycosides: quercitrin (*logP* = 0.644) and rutin (*logP* = -1.063). The results obtained evidently show that the hydrophobicity of the flavonoids under study has a significant effect on their binding to human serum albumin.

3.3.4 Determination of the binding site and interaction forces between flavonoids and HSA

Beside the determination of binding constants, a specific binding site on the protein molecule was identified based on competitive experiments with site-specific markers. The thermodynamic parameters were also calculated and the main acting forces were further characterized for the complexes investigated.

The binding and transport functions of human serum albumin are largely due to the presence of two major hydrophobic binding sites commonly named as Sudlow's site I and II. The determination of specific binding sites is usually

based on a competitive binding, in case of which the ligand under study and the site marker compete for the same binding site on a protein molecule. Thus, compounds with a high binding affinity and selectivity towards definite binding regions on the protein molecule were used for further experiments as the site markers: viz. warfarin (K = 1.2-7.1 $\times 10^5$ M^{-1} to site I on HSA) and ibuprofen (K = 7.1-35.6 $\times 10^5$ M^{-1} to site II on HSA)[98]. The effect of displacement was evaluated by comparing the percentage of binding (Eq. 8) of ligand to protein before and after the addition of the site marker.

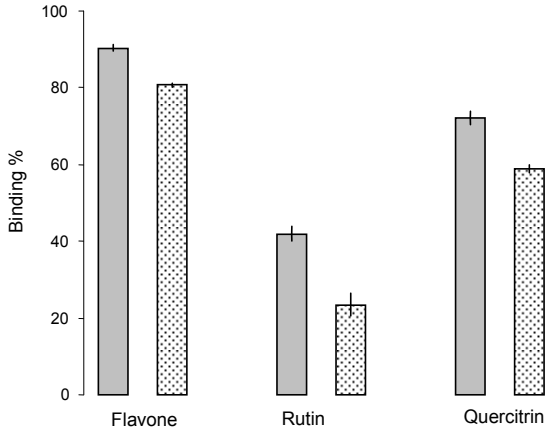

Figure 12. The representation of a competitive binding between flavonoids and warfarin ▭ flavonoid + HSA; ▭ flavonoid + HSA + warfarin.

As demonstrated in Figure 12, the addition of the site I specific compound to the flavonoid-HSA complex resulted in a remarkable reduction in the binding percentage for all of the flavonoids. The decrease was significantly greater (from 41.8 to 23.4 %) for rutin than for quercitrin, which demonstrated a reduction in the binding percentage from 72.1 to 58.8 %, and for flavones, from 90.4 to 80.7 %. This implied that there was a competition between flavonoids and warfarin when both existed in the human serum albumin solution at the same time. Further, ibuprofen was tested as the site II marker, but no marked changes in the binding properties were observed. Experiments demonstrated that ibuprofen did not share the same binding site with flavonoids and independently bound to site II under the conditions studied. Based on these results, it was suggested that studied flavonoids bind to HSA at site I. Moreover, it was noted that the glycosylation of flavonoids had no influence on the binding position.

The binding competition between two ligands typically reduces their affinityies towards protein due to a partial displacement and/or alteration of the protein structure. Under real physiological conditions, the binding of flavonoid to protein could be affected by the presence of a variety of metabolites or drugs

having a similar or higher affinity towards the same binding site on protein. For example, some widely used drugs such as phenylbutazone, salicylamid, p-nitrophenol and quinidine had high *K* values in the range of from 10^4 to 10^5 M^{-1} to the HSA binding site I [98]. Thus, a competitive binding could be supposed and it could alter the binding of flavonoids to proteins, so the real amount of the active fraction of flavonoids in the plasma may be different from that expected.

In order to evaluate the predominant interaction forces between the tested flavonoids and HSA, the binding constants were estimated at two different temperatures (25 and 36.5 °C) and the thermodynamic parameters dependent on temperature were calculated according to Equations (9) and (10). The data obtained is summarized in Table 5.

Table 5. The binding constants measured at various temperatures and thermodynamic parameters for flavonoid-HSA complexes

Compound	T (K)	K ($\times 10^3$ M^{-1})	ΔG (kJ mol^{-1})	ΔH (kJ mol^{-1})	ΔS (J $mol^{-1}K^{-1}$)
Flavone	298	26.88 ± 1.26	-25.3	-15.3	33.6
	309.5	21.39 ± 0.55	-25.7		
Quercitrin	298	7.96 ± 0.18	-22.3	-12.1	34.1
	309.5	6.64 ± 0.10	-22.6		
Rutin	298	5.02 ± 0.14	-21.1	-13.0	27.2
	309.5	4.13 ± 0.16	-21.4		

According to the data presented in Table 5, the flavonoid-HSA complex formation was an exothermic reaction ($\Delta H<0$). The negative value of the free energy (ΔG) indicated the spontaneity of interaction processes, which increased with increasing temperature.

The main acting forces between small molecules and proteins are the hydrogen bonding, van der Waals forces, and electrostatic and hydrophobic interactions. Ross with Subramanian[106] and Leckband[107] characterized the sign and magnitude of the thermodynamic parameters related to various protein associations. Thus, the positive entropy change ΔS is used as an evidence of hydrophobic interactions. Moreover, the dependence of binding constants on the lipophilic characteristics of the compounds under study has been previously demonstrated. Rhamnose contains a non-polar methyl group, as can be seen from its structure (Figure 11), and can contribute to the hydrophobic interactions between glycosides and HSA as well. On the other hand, specific electrostatic interactions are characterized by positive ΔS and negative ΔH

values. The electrostatic interactions could be expected for rutin and quercitrin due to the presence of partially dissociated hydroxyl groups under the conditions used. Consequently, it could be assumed that, besides hydrophobic forces, electrostatic interactions play a considerable role in the formation and maintenance of the stability of the complex between HSA and the flavonoids under study.

The above results confirmed that the CE-FA method is appropriate for the study of ligand-protein interactions. It could be successfully applied to the pharmacokinetic studies of not only new drugs but also naturally occurring bioactive compounds. Taking into account the simple and rapid procedure, which requires small sample volumes and has the potential for automatization, the CE-FA was established to be a valuable support or even an alternative to traditional techniques. Moreover, providing a lot of useful parameters for comprehensive characterization of interactions can considerably extend the utility of the CE-FA method.

4 CONCLUSIONS

The studies performed within the present thesis highlight the potential of capillary electrophoresis for the analysis of proteins. To prove the method's appropriateness and versatility the new column material for separation and fractionation approach to the identification of proteins was demonstrated. Also, the protein-ligand binding were thoroughly characterized by means of the CE frontal analysis.

The results of the present study can be summarized as follows:

- The investigations confirmed the possibility of application of polyether ether ketone as an alternative capillary material to the conventional fused-silica for the CE analysis. Due to the utility of the contactless conductivity detector, the main limitations of the PEEK capillary as the lack of optical transparency and the barest necessity in construction of complicated detection cell were overcome. At the same time, the ability to measure UV-inactive compounds was realized. The negligible technical drawback was exposed during the installation of the capillary into the detection cell because of the mechanical softness of the material.
- The naturally low electroosmotic flow and stability at an extreme pH of the PEEK capillary enabled the use of significantly simplified CE protocols for the analysis of proteins, peptides and other analytes of biological origin. A comparison between the fused-silica and polymer capillaries used for the analysis of model mixtures demonstrated the efficiency and reproducibility of the latter to be better in case of distinct applications, while the separation of proteins was compromised probably by the hydrophobicity of the PEEK material. Summarizing all the positive aspects, the combination of CE–C^4D with the PEEK capillary is considered as a potential candidate for CE-based portable, point-of-care devices to analyze *in situ* samples of biological interest.
- The simple and efficient CE-based fraction collection system for the off-line coupling with a mass spectrometer was developed, whose performance is not tailored to a specific MS instrument. It was demonstrated that the known problems with an off-line CE–MS analysis could be tolerated in addition to the more flexible optimization of the experimental conditions for both systems and the free choice of appropriate stand-alone MS instruments for the study of collected fractions.
- The off-line coupling of the genetic fraction collector with the MALDI-TOF mass spectrometer was verified for the analysis of the proteins mixture. The excellent performance of CE separation and implementation of a droplet counter for a more precise correlation of the fraction composition with the detector signal event resulted in an efficient methodology for peak

identification via MS analysis. The results obtained confirmed the functionality of the proposed set-up design.

- The capillary electrophoresis frontal analysis proved to be a persuasive technique for the investigation of protein-ligand interactions under near-physiological conditions. The binding constants obtained by CE-FA were in conformity with the results of fundamental methods despite the difference in measurement principles. The applicability of the method was focused on the investigation of the binding properties of structurally different bioactive compounds to human serum albumin. A special discussion of the relationship between the structural specificities and binding properties could afterwards facilitate a better understanding of the transportation mechanism and predict the physiological behaviour of ligands guided by the structural features of the latter.
- The approach tested enables the identification of binding sites on a protein molecule based on displacement experiments and, furthermore, the determination of the thermodynamic parameters of binding processes providing information about the main acting forces for the complexes under study. In view of these facts, the study significantly contributed to the potential of CE-FA for an extensive evaluation and characterization of a variety of binding parameters.

REFERENCES

[1] A. Manz, N. Pamme, D. Iossifidis. Bioanalytical chemistry. Imperial College Press, London, 2004.
[2] G. Walsh. Proteins: Biochemistry and Biotechnology. J. Wiley & sons, Chichester, UK, 2002.
[3] D.C. Liebler. Introduction to Proteomics: Tools for the New Biology. Humana Press, Totowa NJ, 2002.
[4] M.R. Wilkins, C. Pasquali, R.D. Appel, K. Ou, O. Golaz, J.-C. Sanchez, J.X. Yan, A.A. Gooley, G. Hughes, I. Humphery-Smith, K.L. Williams, D.F. Hochstrasser. From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and arnino acid analysis. – *Biotechnology* 1996, 14, 61–65.
[5] C.G. Gourin, W. Zhi, B.-L. Adam. Proteomic identification of serum biomarkers for head and neck cancer surveillance. – *Laryngoscope* 2009, 119, 1291-1302.
[6] J. Barry, C.M. Liégeois, A. Janoshazi. Protein kinase C as a peripheral biomarker for Alzheimer's disease. – *Ex.p. Gerontol.* 2010, 45, 64-69.
[7] G. Niccoli, G. Ferrante, N. Cosentino, M. Conte, F. Belloni, M. Marino, M. Baca, R.A. Montone, V. Sabato, D. Schiavino, G. Patriarca, F. Crea. Eosinophil cationic protein: A new biomarker of coronary atherosclerosis. – *Atherosclerosis* 2010, 211, 606-611.
[8] M. Pretolani. Chitiniase-like protein, a novel biomarker of severe asthma. – *Medicine Sciences* 2008, 24, 235-237.
[9] J.E. Noble, M.J. Bailey. Quantitation of protein. – *Methods Enzymol.* 2009, 463, 73-95.
[10] M.H. Simonian, J.A. Smith. Spectrophotometric and colorimetric determination of protein concentration. – *Curr. Protoc. Mol. Biol.* 2006, 10:1A.
[11] C.V. Sapan, R.L. Lundblad, N.C. Price. Colorimetric protein assay techniques. – *Biotechnol. Appl. Biochem.* 1999, 29, 99-108.
[12] H.J. Issaq. The role of separation science in proteomics research. – *Electrophoresis* 2001, 22, 3629-3638.
[13] Y. Shen, R.D. Smith. Proteomics based on high-efficiency capillary separations. – *Electrophoresis* 2002, 23, 3106-3124.
[14] Y. Shi, R. Xiang, C. Horvath, J.A Wilkins. The role of liquid chromatography in proteomics. – *J. Chromatogr. A* 2004, 1053, 27-36.
[15] T. Rabilloud. Two-dimensional gel electrophoresis in proteomics: old, old fashioned, but it still climbs up the mountains. – *Proteomics* 2002, 2, 3-10.
[16] D.E. Garfin. Two-dimensional gel electrophoresis: an overview. – *Trends Anal. Chem.* 2003, 22, 263-272.
[17] Y.-F. Huang, C.-C. Huang, C.-C. Hu, H.-T. Chang. Capillary electrophoresis-based separation techniques for the analysis of proteins. – *Electrophoresis* 2006, 27, 3503-3522.
[18] T. Manabe. Capillary electrophoresis of proteins for proteomic studies. – *Electrophoresis* 1999, 20, 3116-3121.
[19] B. Domon, R. Aebersold. Mass spectrometry and protein analysis. – *Science* 2006, 312, 212-217.
[20] M. Mann, R.C. Hendrickson, A. Pandey. Analysis of proteins and proteomes by mass spectrometry. – *Annu. Rev. Biochem.* 2001, 70, 437-473.
[21] E. Gelpi. Interfaces for coupled liquid-phase separation/mass spectrometry techniques. An update on recent developments. – *J. Mass Spectrom.* 2002, 37, 241-253.

[22] D.C. Kim, D.J. Kang. Molecular recognition and specific interactions for biosensing applications. – *Sensors* 2008, 8, 6605-6641.
[23] B. Strehlitz, N. Nikolaus, R. Stoltenburg. Protein detection with aptamer biosensors. – *Sensors* 2008, 8, 4296-4307.
[24] J. Hirose, Y. Murakami-Yamaguchi, M. Ikeda, H. Narita. Determination of the major food allergen, ovomucoid, with an oligoclonal enzyme-linked immunosorbent assay. – *Animal Cell Techn.: Basic&Appl. Aspects* 2006, 3, 317-323.
[25] J.M. Rolland, E. Apostolou, M.P. de Leon, C.S. Stockley, R.E. O'Hehir. Specific and sensitive enzyme-linked immunosorbent assay for analysis of residual allergenic food proteins in commercial bottled wine fined with egg white, milk, and nongrape-derived tannins. – *J. Agric. Food Chem.* 2008, 56, 349-354.
[26] W. Xia, T. Yang, G. Shankar, I.M. Smith, Y. Shen, D.M. Walsh, D.J. Selkoe. A specific enzyme-linked immunosorbent assay for measuring β-amyloid protein oligomers in human plasma and brain tissue of patients with Alzheimer disease. – *Arch. Neurol.* 2009, 66,190-199.
[27] R.D. Saville, N.T. Constantine, F.R. Cleghorn, N. Jack, C. Bartholomew, J. Edwarks, P. Gomez, W.A. Blattner. Fourth-generation enzyme-linked immunosorbent assay for the simultaneous detection of human immunodeficiency virus antigen and antibody. – *J. Clin. Microbiol.* 2001, 39, 2518-2524.
[28] R.B. Wong, Z.H. Ahmed. Development of an enzyme-linked immunosorbent assay for imazaquin herbicide. – *J. Agric. Food. Chem.* 1992, 40, 811-816.
[29] M. Nagahama, K. Kobayashi, S. Ochi, J. Sakurai. Enzyme-linked immunosorbent assay for rapid detection of toxins from *Clostridium perfringens*. – *FEMS Microbiol. Letters* 1991, 84, 41-44.
[30] H.-L. Liu, J.-P. Hsu. Recent developments in structural proteomics for protein structure determination. – *Proteomics* 2005, 5, 2056-2068.
[31] R. Matthiesen (editor). Mass spectrometry data analysis in proteomics. Humana Press, Totowa, NJ, 2008.
[32] M.R. Larsen, P. Roepstorff. Mass spectrometric identification of proteins and characterization of their post-translational modifications in proteome analysis. – *Fresenius J. Anal. Chem.* 2000, 366, 677-690.
[33] K. Wüthrich. Protein structure determination in solution by NMR spectroscopy. – *J. Biol. Chem.* 1990, 256, 22059-22062.
[34] A. Barth. Infrared spectroscopy of proteins. – *Biochim.Biphys. Acta* 2007, 1767, 1073-1101.
[35] U. Aebi, W.E. Fowler, P.R. Smith. Three-dimensional structure of proteins determined by electron microscopy. – *Ultramicroscopy* 1982, 8, 191-206.
[36] U. Aebi, W.E. Fowler, E.L. Buhle, P.R. Smith. Electron microscopy and image processing applied to the study of protein structure and protein-protein interactions. – *J. Ultrastruct. Res.* 1984, 88, 143-176.
[37] A. Ilari, C. Savino. Protein structure determination by x-ray crystallography. – *Methods Mol. Biol.* 2008, 452, 63-87.
[38] M. Mann, O.L. Jensen. Proteomic analysis of post-translational modifications. – *Nature Biotech.* 2003, 21, 255-261.
[39] J.P. Goddard, J.-L. Reymond. Recent advances in enzyme assays. – *Trends in Biotehn.* 2004, 22, 363-370.
[40] J. Bao, F.E. Regnier. Ultramicro enzyme assays in a capillary electrophoretic system. – *J. Chromatogr. A* 1992, 608, 217-224.

[41] M.L. Howard, J.J. Hill, G.R. Galluppi, M.A. McLean. Plasma protein binding in drug discovery and development. – *Comb. Chem. Throughput Screen.* 2010, 13, 170-187.
[42] K. Vuignier, J. Schappler, J.-L. Veuthey, P.-A. Carrupt, S. Martel. Drug-protein binding: a critical review of analytical tools. – *Anal. Bioanal. Chem.* 2010, 398, 53-66.
[43] N.H.H. Heegaard. Capillary electrophoresis for the study of affinity interactions. – *J. Mol. Recogn.* 1998, 11, 141-148.
[44] J.P. Landers (editor). Handbook of capillary and microchip electrophoresis and associated microtechniques. CRC Press, Boca Raton, FL, 2007.
[45] D. Harvey. Modern Analytical Chemistry. McGraw-Hill Science, Boston, 1999.
[46] V. Rohlicek, Z. Deyl, I. Mikšik. Determination of the isoelectric point of the capillary wall in capillary electrophoresis: application to plastic capillaries. – *J. Chromatogr. A* 1994, 662, 369–373.
[47] S.C.C. Chiang, S.F.Y. Li. Separation of porphyrins by capillary electrophoresis in fused-silica and ethylene vinyl acetate copolymer capillaries with visible absorbance detection. – *Biomed. Chromatogr.* 1997, 11, 366-370.
[48] J. Horvath, V. Dolnik. Polymer wall coatings for capillary electrophoresis. – *Electrophoresis* 2001, 22, 644-655.
[49] I. Rodriguez, S.F.Y. Li. Surface deactivation in protein and peptide analysis by capillary electrophoresis. – *Anal. Chim. Acta* 1999, 383, 1-26.
[50] C.A. Lucy, A.M. MacDonald, M.D. Gulcev. Non-covalent capillary coatings for protein separations in capillary electrophoresis. – *J. Chromatogr. A* 2008, 1184, 81-105.
[51] F. Benavente, V. Sanz-Nebot, J. Barbosa, R. van der Heijden, J. van der Greef, T. Hankemeier. CE-ESI-MS of biological anions in plastic capillaries at high pH. – *Electrophoresis* 2007, 28, 944–949.
[52] P. J. Schneider, H. Engelhardt. Novel applications for organic polymer capillaries. – *J. Chromatogr. A* 1998, 802, 17–22.
[53] J. Tanyanyiwa, S. Leuthardt, P.C. Hauser. Electrophoretic separations with polyether ether ketone capillaries and capacitively coupled contactless conductivity detection. – *J. Chromatogr. A* 2002, 978, 205–211.
[54] M. Macka, W.-C. Yang, P. Zakaria, A. Shitangkoon, E.F. Hilder, P. Andersson, P. Nesterenko, P.R. Haddad. Poly(tetrafluoroethylene) separation capillaries for capillary electrophoresis: properties and applications. – *J. Chromatogr. A* 2004, 1039, 193–199.
[55] L. Miller, M. Juza. Stability problems of polyether ether ketone and ethylene-tetrafluoroethylene copolymer tubing in simulated moving bed operation. – *J. Chromatogr. A* 2005, 1094, 165–168.
[56] M. Lacroix, V. Poinsot, C. Fournier, F. Couderc. Laser-induced fluorescence detection schemes for the analysis of proteins and peptides using capillary electrophoresis. – *Electrophoresis* 2005, 26, 2608–2621.
[57] X. Xu, L. Li, S.G. Weber. Electrochemical and optical detectors for capillary and chip separations. – *Trends Anal. Chem.* 2007, 26, 68-79.
[58] J. Kuijt, F. Ariese, C. Gooijer. Novel optical detection techniques in CE based on phosphorescence or chemiluminescence. – *Compr. Anal. Chem.* 2005, 45, 375-411.
[59] P. Kuban, P.C. Hauser. Fundamentals of electrochemical detection techniques for CE and MCE. – *Electrophoresis* 2009, 30, 3305-3314.
[60] A.J. Zemann. Conductivity detection in capillary electrophoresis. – *Trends Anal. Chem.* 2001, 20, 346-354.
[61] T. Kappes, B. Galliker, M.A. Schwarz, P.C. Hauser. Portable capillary electrophoresis instrument with amperometric, potentiometric and conductometric detection. – *Trends Anal. Chem.* 2001, 20, 133-139.

[62] A. Seiman, J. Martin, M. Vaher, M. Kaljurand. A portable capillary electropherograph equipped with a cross-sampler and a contactless-conductivity detector for the detection of the degradation products of chemical warfare agents in soil extracts. – *Electrophoresis* 2009, 30, 507-514.
[63] D.M. Osbourn, D.J. Weiss, C.E. Lunte. On-line preconcentration methods for capillary electrophoresis. – *Electrophoresis* 2000, 21, 2768-2779.
[64] M. Graf, R.G. Garćia, H. Wätzig. Protein adsorption in fused-silica and polyacrylamide-coated capillaries. – *Electrophoresis* 2005, 26, 2409-2417.
[65] D. Schmalzing, C.A. Piggee, F. Foret, E. Carrilho, B.L. Karger. Characterization and performance of a neutral hydrophilic coating for the capillary electrophoretic separation of biopolymers. – *J. Chromatogr. A* 1993, 652, 149-159.
[66] M. Gilges, M.H. Kleemiss, G. Schomburg. Capillary zone electrophoresis separations of basic and acidic proteins using poly(vinyl alcohol) coatings in fused silica capillaries. – *Anal. Chem.* 1994, 66, 2038-2046.
[67] D. Corradini. Buffer additives other than the surfactant sodium dodecyl sulfate for protein separations by capillary electrophoresis. – *J. Chromatogr. B* 1997, 699, 221-256.
[68] M. Moini, S.M. Demars, H. Huang. Analysis of carbonic anhydrase in human red blood cells using capillary electrophoresis/electrospray ionization-mass spectrometry. – *Anal. Chem.* 2002, 74, 3772-3776.
[69] S. Amon, A. Plemati, A. Rizzi. Capillary zone electrophoresis of glycopeptides under controlled electroosmotic flow conditions coupled to electrospray and matrix-assisted laser desorption/ionization mass spectrometry. – *Electrophoresis* 2006, 27, 1209-1219.
[70] B. Miralles, V. Rothbauer, M.A. Manso, L. Amigo, I. Krause, M. Ramos. Improved methods for the simultaneous determination of whey proteins, caseins and *para*-κ-casein in milk and dairy products by capillary electrophoresis. – *J. Chromatogr. A* 2001, 915, 225-230.
[71] D.C. Simpson, R.D. Smith. Combining capillary electrophoresis with mass spectrometry for applications in proteomics. – *Electrophoresis* 2005, 26, 1291-1305.
[72] F.E. Ahmed. The role of capillary electrophoresis-mass spectrometry to proteome analysis and biomarker discovery. – *J. Chromatogr. B* 2009, 877, 1963-1981.
[73] D.R. Zalewski, S. Schlautmann, R.B.M. Schasfoort, H.J.G.E. Gardeniers. Electrokinetic sorting and collection of fractions for preparative capillary electrophoresis on a chip. – *Lab Chip* 2008, 8, 801-809.
[74] H. Stutz. Advances in the analysis of proteins and peptides by capillary electrophoresis with matrix-assisted laser desorption/ionization and electrospray-mass spectrometry detection. – *Electrophoresis* 2005, 26, 1254-1290.
[75] K.L. Walker, R.W. Chiu, C.A. Monnig, C.L. Wilkins. Off-line coupling of capillary electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. – *Anal. Chem.* 1995, 67, 4197-4204.
[76] C.W. Huck, R. Bakry, L.A. Huber, G.K. Bonn. Progress in capillary electrophoresis coupled to matrix-assisted laser desorption/ionization - time of flight mass spectrometry. – *Electrophoresis* 2006, 27, 2063-2074.
[77] M. Lechner, A. Seifner, A.M. Rizzi. Capillary isoelectric focusing hyphenated to single- and multistage matrix-assisted laser desorption/ionization mass spectrometry using automated sheath-flow-assisted sample deposition. – *Electrophoresis* 2008, 29, 1974-1984.
[78] M. Minarik, F. Foret, B.L. Karger. Fraction collection in micropreparative capillary electrophoresis and capillary isoelectric focusing. – *Electrophoresis* 2000, 21, 247-254.

[79] G. Choudhary, J. Chakel, W. Hancock, A. Torres-Duarte, G. McMahon, I. Wainer. Investigation of the potential of capillary electrophoresis with off-line matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for clinical analysis: examination of a glycoprotein factor associated with cancer cachexia. – *Anal. Chem.* 1999, 71, 855-859.
[80] S.I. Snovida, V.C. Chen, O. Krokhin, H. Perreault. Isolation and identification of sialylated glycopeptides from bovine α_1-acid glycoprotein by off-line capillary electrophoresis MALDI-TOF mass spectrometry. – *Anal. Chem.* 2006, 78, 6556-6563.
[81] T.A. Crowley, M.A. Hayes. Analysis of human blood serum using the off-line coupling of capillary isoelectric focusing to matrix-assisted laser desorption/ionization time of flight mass spectrometry. – *Proteomics* 2005, 5, 3798-3804.
[82] F. Zhou, M.V. Johnston. Protein characterization by on-line capillary isoelectric focusing, reversed-phase liquid chromatography, and mass spectrometry. – *Anal. Chem.* 2004, 76, 2734-2740.
[83] X. Fang, L. Yang, W. Wang, T. Song, C.S. Lee, D.L. DeVoe, B.M. Balgley. Comparison of electrokinetics-based multidimensional separations coupled with electrospray ionization-tandem mass spectrometry for characterization of human salivary proteins. – *Anal. Chem.* 2007, 79, 5785-5792.
[84] C.S. Effenhauser, A. Manz, H.M. Widmer. Manipulation of sample fractions on a capillary electrophoresis chip. – *Anal. Chem.* 1995, 67, 2284-2287.
[85] Z. Glatz. Determination of enzymatic activity by capillary electrophoresis. – *J. Chromatogr. B* 2006, 841, 23-37.
[86] A. Schuchert-Shi, P.C. Hauser. Monitoring the enzymatic conversion of urea to ammonium by conventional or microchip capillary electrophoresis with contactless conductivity detection. – *Anal. Biochem.* 2008, 376, 262-267.
[87] J. Jitkova, C.N. Carrigan, C.D. Poulter, S.N. Krylov. Monitoring the tree enzymatic activities involved in posttranslational modifications of Ras proteins. – *Anal. Chim. Acta* 2004, 521, 1-7.
[88] M. Kulp, M. Kaljurand, T. Käämbre, P. Sikk, V. Saks. *In situ* monitoring of kinetics of metabolic conversion of ATP to ADP catalyzed by MgATPases of muscle Gastrocnemius skinned fibers using micellar electrokinetic chromatography. – *Electrophoresis* 2004, 25, 2996-3002.
[89] M. Kulp, M. Vaher, M. Kaljurand. Miniaturization of sampling for chemical reaction monitoring by capillary electrophoresis. – *J. Chromatogr. A* 2005, 1100, 126-129.
[90] P. Kuban, B. Karlberg. Flow/sequential injection sample treatment coupled to capillary electrophoresis. – *Anal. Chim. Acta* 2009, 648, 129-145.
[91] S. Nováková, S.V. Dyck, A.V. Schepdael, J. Hoogmartens, Z. Glatz. Electrophoretically mediated microanalysis. – *J. Chromatogr. A* 2004, 1032, 173-184.
[92] J. Zhang, J. Hoogmartens, A.V. Schepdael. Advances in CE-mediated microanalysis: an update. – *Electrophoresis* 2008, 29, 56-65.
[93] Y. Tanaka, S. Terabe. Estimation of binding constants by capillary electrophoresis. – *J. Chromatogr. B* 2002, 768, 81–92.
[94] H. Kajiwara. Affinity capillary electrophoresis of proteins and peptides. – *Anal. Chim. Acta* 1999, 383, 61-66.
[95] J. Zavaleta, D. Chinchilla, A. Brown, A. Ramirez, V. Calderon, T. Sogomonyan, F.A. Gomez. Recent developments in affinity capillary electrophoresis: a review. – *Curr. Anal. Chem.* 2006, 2, 35-42.
[96] A.R. Timerbaev, A.V. Rudnev, O. Semenova, C.G. Hartinger, B.K. Keppler. Comparative binding of antitumor indazolium [*trans*-tetrachlorobis(1*H*-

indazole)ruthenate(III)] to serum transport proteins assayed by capillary zone electrophoresis. – *Anal. Biochem.* 2005, 341, 326–333.
[97] J. Østergaard, N. Heegaard. Capillary electrophoresis frontal analysis: Principles and applications for the study of drug-plasma protein binding. – *Electrophoresis* 2003, 24, 2903-2913.
[98] J. Østergaard, C. Schou, C. Larsen, N. Heegaard. Evaluation of capillary electrophoresis-frontal analysis for the study of low molecular weight drug-human serum albumin interactions. – *Electrophoresis* 2002, 23, 2842-2853.
[99] K. Vuignier, J. Schappler, J.-L. Veuthey, P.-A. Carrupt, S. Martel. Improvement of a capillary electrophoresis/frontal analysis (CE/FA) method for determing binding constants: Discussion on relevant parameters. – *J. Pharm. Biomed. Anal.* 2010, 53, 1288-1297.
[100] Molinspiration Cheminformatics miLogP version 2.2., http://www.molinspiration.com , accessed Sept 2009.
[101] K. Lemanska, H. Szymusiak, B. Tyrakowska, R. Zieliski, A. Soffers, I. Rietjens. The influence of pH on antioxidant properties and the mechanism of antioxidant action of hydroxyflavones. – *Free Radical Biol. Med.* 2001, 31, 869-881.
[102] N.N. Luan, J.X. Wu, Y.M. Song, Q. Wu. Thermodynamic studies on the binding of rutin and serum albumin. – *Guang Pu Xue Yu Guang Pu Fen Xi* 2008, 28, 856-859.
[103] S. Bi, L. Ding, Y. Tian, D. Song, X. Zhou, X. Liu, H. Zhang. Investigation of the interaction between flavonoids and human serum albumin. – *J. Molec. Struct.* 2004, 703, 37-45.
[104] Q. Lu, C. Ba, D. Chen. Investigating noncovalent interactions of rutin - serum albumin by capillary electrophoresis - frontal analysis. – *J. Pharm. Biomed. Anal.* 47, 888-891.
[105] A. Diniz, L. Escuder-Gilabert, N.P. Lopes, R.M. Villanueva-Camanas, S. Sagrado, M.J. Medina-Hernandez. Characterization of interactions between polyphenolic compounds and human serum proteins by capillary electrophoresis. – *Anal. Bioanal. Chem.*2008, 391, 625-632.
[106] D.P. Ross, S. Sabramanian. The thermodynamics of protein association reactions: forces contributing to stability. – *Biochemistry* 1981, 20, 3096-3102.
[107] D. Leckband. Measuring the forces that control protein interactions. – *Annu. Rev. Biophys. Biomol. Struct.* 2000, 29, 1-26.

ACKNOWLEDGMENTS

The research presented in this thesis has been carried out at the Chair of Analytical Chemistry of the Department of Chemistry at Tallinn University of Technology.

Here, I would like to thank all the people who assisted and encouraged me in various ways during the PhD study and at the time of preparing this work.

Foremost, I wish to express my deepest gratitude to my supervisor, Professor Mihkel Kaljurand, for his great assistance and expert guidance in the field of analytical chemistry. His advanced ideas and useful suggestions were valuable for research, as well as discussions were incentive and effective. I am sincerely grateful for giving confidence to work in his research group.

I am indebted to my colleagues for their advices and useful comments, for help with experimental setup and all types of technical problems, and of course for positive and friendly atmosphere. I am grateful to Maria Kulp, who taught me the basis of capillary electrophoresis at the beginning of the study. Thank you, Tatjana, Natalja, Maria, Eeva-Gerda, Andrus, Anna-Liisa, Kati, Merike. It was a pleasure to share studies and life with such wonderful people, among whom I have good friends now. My special words of thanks belong to my close friend, Jelena, for her ability to be always cheerful and encouraging, both personally and professionally.

Самые теплые слова благодарности моей семье. Я искренне признательна своей маме за ее неиссякаемый жизненный оптимизм, безграничную поддержку и безоговорочную веру в меня. Спасибо мужу и доченьке за подаренное светлое чувство радости, тепла и счастья, за их одобрение и понимание.

Finally, this work was financially supported by the Estonian Science Foundation and the Estonian Ministry of Research and Education. The European Social Fund's Doctoral Studies and Internationalisation Programme DoRa activity 8 "Supporting the Participation of Young Researchers in the International Exchange of Knowledge" is also acknowledged.

ABSTRACT

Nowadays, the utility of capillary electrophoresis has been vastly expanded to various research fields. In order to make CE more attractive for proteomic applications a diversity of CE systems and operation modes are currently suggested, which exploited different physicochemical protein properties for their analysis and vice versa. Nevertheless, novel applications may still contribute to the area of proteomics. The main goals of the present work were related to the CE-based method development describing novel separation approaches and elaborating advanced instrumental design. Furthermore, the utility of the proposed systems was demonstrated placing main emphasis on the analysis of proteins.

This study explored the possibility of application of a polymeric PEEK capillary to the CE separation. The main drawback as the lack of optical transparency was eliminated owing to the use of the C^4D detector. The enhanced performance of the PEEK capillary, in comparison with fused-silica, was achieved for selected applications, whereas the reproducibility of the results was detrimental to the protein and therefore had to be discussed. Furthermore, in view of the low electroosmotic flow the fast biological anions were analyzed applying opposite end injections without compromising separation and using simple experimental conditions. Thus, the combination of CE–C^4D with the PEEK capillary could be preferable to *in situ* analysis on portable instrument. Another approach developed here employs CE for fraction collection with a subsequent MS-based analysis. An important strive to realize identification tool competitive to an on-line CE-MS involved the design of a robust CE fractionation system based on the sheath liquid flow configuration and development of a precise protocol. Besides, the results of an effective application of capillary electrophoresis in conjunction with the MALDI-TOF-MS to the analysis of the proteins mixture were presented. Accordingly, the proposed off-line CE-MS approach enabled a protein to be purified from an extremely complex biological environment, in addition to the availability of various intermediate manipulations and proper choice of stand-alone MS techniques.

In the last section, the application of CE in the format of frontal analysis was utilized for one of the most addressed issues in proteomics - the characterization of interactions between proteins and bioactive compounds. The binding constants obtained by CE-FA were comparable with literature data and demonstrated their evident dependence on the structural pattern of ligand. In view of the fact that the majority of conventional techniques are limited to the determination of binding constants, this study illustrated that the characterization of acting forces and determination of the binding site can be accurately accomplished by using the same approach to a more thorough investigation of binding properties.

KOKKUVÕTE

Tänapäeval on kapillaarelektroforeesi (CE) kasutamine laienenud paljudele erinevatele uurimisaladele. Et muuta CE meetodit atraktiivsemaks ka proteoomika rakendustes, on selles dissertatsioonis välja pakutud mitmeid erinevaid uusi lahendusi valkude kapillaarelektroforeetiliseks lahutamiseks ja uurimiseks.

Tehtud uuringud olid seotud polümeerse PEEK kapillaari võimaliku rakendusega kapillaarelektroforeetilisel lahutamisel. Töös tõestati, et nende peamisel puudusel – optilisel läbipaistmatusel – ei ole enam tähtsust pärast kontaktita juhtivusdetektori kasutusele võtmist. PEEK kapillaari tõhusus võrreldes kvartskapillaariga saavutati peptiidide ja dikarboksüülhapete jaoks, samal ajal kui valkude puhul oli tulemuste korratavus ebapiisav ja seepärast vajab PEEK kapillaari kasulikkus veel uurimist. Peale selle olid nõrga elektroosmootse voo tõttu kiired bioloogilised anioonid efektiivselt analüüsitavad, kasutades vastassuunalist sisestamist ja lihtsaid eksperimentaalseid tingimusi. Seega, kapillaarelektroforeesi ja juhtivusdetektori kombinatsioon PEEK kapillaariga võib olla eelistatav *in situ* analüüsil portatiivse instrumendiga. Teine väljatöötatud valkude analüüsi meetod kasutab kapillaarelektroforeesi fraktsioonide kogumist, millele järgneb nende edasine mass-spektromeetriline analüüs. Püüe luua identifitseerimise vahend, mis oleks konkurentsivõimeline *on-line* CE-MS-ga, realiseerus antud töös lihtsa fraktioneerimise süsteemi kavandamisega ja täpse protokolli väljatöötamisega. Töös on esitatud ka selle süsteemi efektiivne rakendus valkude segu analüüsiks. Seega võimaldab pakutud *off-line* CE-MS lähenemisviis esiteks puhastada valke keerulistest bioloogilisest maatriksitest, teiseks kasutada erinevaid vahepealseid manipulatsioone kogutud fraktsioonidega ja lõpuks valida sobiv mass-spektromeeter komponentide identifitseerimiseks.

Töö viimases osas kasutati kapillaarelektroforeesi frontaalset analüüsi (CE-FA) valkude ja bioaktiivsete ühendite vaheliste vastastikmõjude iseloomustamiseks, mis on üks enim uuritav proteoomika valdkond. CE-FA meetodiga saadud sidumiskonstandid olid sarnased kirjanduse andmetega ning demonstreerisid silmnähtavat sõltuvust ligandi struktuurist. Peale selle võimaldab nimetatud meetod iseloomustada toimivaid molekulidevahelisi interaktsioone ja teha kindlaks sidumiskohta. Pidades silmas, et enamik traditsiooniliselt kasutatavatest meetoditest on piiritletud vaid sidumiskonstantide määramisega, näitas see töö, et CE-FA sobib hästi sidumisomaduste mitmekülgseks analüüsiks

ORIGINAL PUBLICATIONS

PUBLICATION I

T. Knjazeva, M. Kulp, M. Kaljurand. CE separation of various analytes of biological origin using polyether ether ketone capillaries and contactless conductivity detection. Electrophoresis 2009, 30, 424-430.

Tatjana Knjazeva
Maria Kulp
Mihkel Kaljurand

Department of Chemistry, Tallinn Technical University, Tallinn, Estonia

Received April 18, 2008
Revised July 16, 2008
Accepted August 14, 2008

Research Article

CE separation of various analytes of biological origin using polyether ether ketone capillaries and contactless conductivity detection

The development of efficient and sensitive analytical methods for the separation, identification and quantification of complex biological samples is continuously a topic of high interest in biological science. In the present study, the possibility of using a polyether ether ketone (PEEK) capillary for the CE separation of peptides, proteins and other biological samples was examined. The performance of the tubing was compared with that of traditional silica capillaries. The CE analysis was performed using contactless conductivity detection (C^4D), which eliminated any need for the detection window and was suitable for the detection of optically inactive compounds. In the PEEK capillary the cathodic EOF was low and of excellent stability even at extremes pH. In view of this fast biological anions were analyzed using an opposite end injection technique without compromising separation. A comparison of the performances of fused-silica and polymer capillaries during the separation of model sample mixtures demonstrated the efficiency and separation resolution of the latter to be higher and the reproducibility of the migration times and peak areas is better. Furthermore, PEEK capillaries allowed using simple experimental conditions without any complicated modification of the capillary surface or use of an intricate buffer composition. The PEEK capillaries are considered as an attractive alternative to the traditional fused-silica capillaries and may be used for the analysis of complex biological mixtures as well as for developing portable devices.

Keywords:
Complex biological samples / Contactless conductivity detection / Polyether ether ketone capillary
DOI 10.1002/elps.200800252

1 Introduction

The capillary is a key element of the CE instrument where the separation takes place. Moreover, the rate of the EOF depends on the material characteristics of the capillary. The capillary should be made of chemically and electrically inert, flexible and robust material. One should not neglect the cost of the material either. Satisfying most of these requirements, fused silica is the dominant material of the capillaries used in CE. Moreover, fused-silica capillaries enable the detection below 200 nm up through the visible spectrum to be used [1]. Thus, commercial CE instruments equipped with optical detectors have been most widely used up to our time. Finally, the properties of the surface of the fused-silica capillary have thoroughly been studied, permitting varying the EOF in the desirable manner [2–5]. As a consequence, CE separations are carried out using mostly (close to 100%) fused-silica capillaries. The main drawback of the latter is the challenging separation of biological samples, especially peptides and proteins. Problems arise from the electrostatic interactions between the positively charged proteins and negatively charged silanol groups on the inner capillary wall, leading to the loss of efficiency, poor reproducibility of migration times, low protein recovery and decreased sensitivity [6–8]. To overcome protein absorption on the capillary inner wall, a variety of techniques have been developed such as the use of electrolyte solutions at high pH values [7], as well as dynamic and static coating [9–13]. However, the coatings are stable only in a certain range of pH values; many of additives may cause a decrease in detection signals, incompatibility with the mass-spectrometric detection and interactions with proteins.

A comparison has shown the optical, mechanical and thermal properties of polymers to be inferior to those of

Correspondence: Tatjana Knjazeva, Department of Chemistry, Tallinn Technical University, Akadeemia tee 15, Tallinn 12618, Estonia
E-mail: tatjana.knjazeva@yahoo.com
Fax: +372-6202828

Abbreviations: C^4D, capacitively coupled contactless conductivity detection**; His,** histidine**; PEEK,** polyether ether ketone

fused silica. However, polymer capillaries could potentially be an attractive alternative to fused silica ones because of the inertness or different surface chemistry of their inner wall. But so far these materials have been rarely used in the production of CE microfluidic devices [14–16]. Over the last ten years only a few papers have been published on the employment of polyether ether ketone (PEEK) [17–19], PMMA [17, 18], PTFE [20, 21] and other organic polymer materials [22, 23] as column material in CE. Because of the organic nature of the surface, the performance of polymer capillaries is completely different from that of silica and has not been thoroughly studied. Macka and co-workers demonstrated the possibility of using PTFE as a capillary in CE [20]. In this study the main problems encountered with the PTFE capillary consisted in that it was mechanically too soft for routine usage and the crystallinity caused a light scattering, leading to high background absorbance values. A PEEK polymer is an exceptionally strong thermoplastic that retains its mechanical properties even at very high temperatures. The material also resists an attack by a wide range of organic and inorganic chemicals and is stable even at extreme pH because of the chemical resistance of backbones [24]. However, the application of the PEEK capillary was still limited due to its lack of optical transparency. Because most organic polymeric capillaries contain UV-absorbing functional groups they are hardly transparent to the UV light below 270 nm. Schneider and Engelhardt used PEEK and PMMA capillaries equipped with an inserted detection window made of a fused-silica capillary for photometric detection [18]. As the construction of a detection cell is rather complicated and the connection has an influence on peak efficiency, this work remained ostentatious.

The contactless conductivity detection (C^4D) is a recent innovation in the field of detection for capillary electrophoretic analysis [25–27]. In the case of this universal detection mode, the difference in conductivity between the separation electrolyte and solute zones is measured. Among the many different applications, conductivity detection has been demonstrated to be useful for the determination of amino acids, peptides and proteins with the detection limit in the micromolar range [28–30]. The inculcation of the C^4D detection mode enables the applications of the PEEK capillary to be significantly increased and its new attractive features to be found. The latest studies in this field is concerned with the use of the PEEK capillary for the separation of cations and anions [19]. Undoubtedly, this work was pioneering in this field although not all the possible potentials of such a combination were dealt with.

The present work stems from several considerations. As follows from the introduction the use of PEEK capillaries has been rare despite the inertness of their inner surface unlike that of fused silica [24]. Transparency to the UV light makes fused silica suitable for the analysis of high molecular compounds, such as humic material or proteins, despite the elaborate procedures necessary for the preparation of the capillary inner surface to avoid adsorption problems. However, in contrast to C^4D, optical detectors are bulky and difficult to miniaturize. This circumstance and the elaborate analysis protocols make it difficult to design CE-based portable, point-of-care detectors that are simple to operate by an unskilled individual and could be used *in situ* for military or medical purposes. The latter applications definitely involve analysis of high molecular weight compounds. In contrast, the PEEK capillary should simplify analysis protocols significantly (there is no need for the washing or use of an expensive pre-coated capillary). The aim of this work was to investigate the possibility of using a combination of CE–C^4D with PEEK capillaries, develop a simplified protocol for the analysis of proteins and humic substances and employ the capillaries for the separation of various analytes of biological origin. In order to verify the utility of the analysis protocols developed for PEEK capillaries, the test mixtures were also analyzed in bare fused-silica capillaries. The results were compared with those obtained with PEEK capillaries. An attempt was made to demonstrate the performance of CE using fused-silica capillaries to be less favorable.

2 Materials and methods

2.1 Instrumentation

All measurements were made using a purpose-made electrophoretic instrument. Electrophoretic separations were carried out at a voltage of from 8 to 19 kV with a security system cutting off the high voltage power supply if the box is opened. The electropherograms were recorded using the contactless conductivity detector *TraceDec*® (I.S.T., Austria) transferred to a personal computer working with special *TraceDecMonitor* software and edited by Matlab (MathWorks, Natick, MA, USA). The samples were introduced electrokinetically by applying voltage at various injection times. All experiments were performed at ambient temperature without temperature control.

In experiments, fused-silica (Polymicro Technologies, USA) and PEEK (Upchurch, Oak Harbor, WA, USA) capillaries with dimensions 75 μm id and 360 μm od were employed. For conditioning, at the beginning of each working day and prior to each change of the running electrolyte the capillary was washed with 0.1 M NaOH for 10 min, deionized water for 10 min and the running buffer for 10 min. To improve the reproducibility of analyses, the capillary was also rinsed with 0.1 M NaOH (3 min), deionized water (3 min) and the running buffer (3 min) between consecutive runs. These standard conditions were used for all capillaries.

2.2 Chemicals, solutions and samples, procedures

All chemicals were obtained as an analytical reagent grade and were used without additional purification. Malonic,

succinic, glutaric, phthalic, adipic and pimelic acids were supplied by Sigma-Aldrich (Steinheim, Germany). The stock solutions of dicarboxylic acids at a concentration of 50 mM were prepared from the corresponding analytes using Milli-Q water. All multicomponent solutions were freshly prepared every day from the corresponding stock solutions by dissolving them in the running buffer.

The HPLC peptide standard mixture H2016, cytochrome c, myoglobin, ribonuclease A, β-lactoglobulin A+B and α-lactalbumin were purchased from Sigma-Aldrich and Fluka (Buchs, Switzerland). The stock solutions of proteins were prepared at a concentration of 10 mg/mL, stored at –20°C and adjusted to room temperature prior to use.

The humic acid used in this work was Fluka HA standard No. 53680 (analysis number 35837/1 293). The sample was prepared at a concentration of 1 mg/mL by dissolving humic acid in Milli-Q water containing 5% NaOH for complete dissolution. For electrophoretic analysis, the stock solution was filtered through a 0.45 μm filter, degassed in an ultrasonic bath and diluted ten times.

Buffers with an ionic strength of 7.5–20 mM were prepared from 2-(*N*-morpholino)ethanesulfonic acid (MES), L-histidine (His) and benzoic acid, which were obtained from Sigma and Fluka. Fresh buffer solutions were prepared daily before use and were used without adjustment at their natural pH values. To improve solubility benzoic acid was heated in the water bath at 65°C for 10 min. Mesityl oxide used as an EOF marker was also from Fluka.

The water used for the preparation of the buffer and samples was obtained from the Milli-Q water purification system (Millipore S.A., Molsheim, France). All the electrolytes and rinsing solutions were filtered before use through 0.45 μm nitrocellulose Millipore filters. pH was measured using a 744 pH Meter (Metrohm, Herisau, Switzerland) with a combined glass electrode. The pH meter was calibrated with an aqueous standard buffer (pH 4.00 and 7.00) from Merck.

3 Results and discussion

3.1 EOF in the PEEK capillary

PEEK has repeating monomers of ketone and two ether groups (Fig. 1) [24]. In the view of its chemical structure, the polymer capillary is theoretically neutral and does not possess charged surface groups. However, we carried out series of experiments and measured the magnitude of the low EOF. Using mesityloxide as an EOF marker, the respective mobility value was $2.65 \pm 0.04 \times 10^{-4}\,cm^2/V\,s$ for the PEEK capillary, with an internal diameter of 75 μm in the case of a 7.5 mM MES/His buffer (pH 6.1). In order to estimate the stability of the PEEK column, the reproducibility of the results was calculated. The EOF was found to be highly reproducible relating to the run-to-run RSD of the apparent marker mobility of 1.4% ($n = 8$). The value obtained well coincided with literature data $2.6 \times 10^{-4}\,cm^2/V\,s$ (10 mM MES/His buffer, pH 6) [19]. At the same conditions, the EOF in the fused-silica capillary was considerably stronger, being $6.42 \times 10^{-4}\,cm^2/V\,s$.

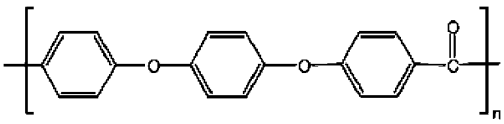

Figure 1. The chemical structure of polyether ether ketone polymer.

3.2 Dicarboxylic acids

The naturally low EOF may potentially have an advantage in the separation of strong anions or species having more than one negatively charged group, for example, dicarboxylic acids. In analytical biochemistry the separation of dicarboxylic acids is an interesting and challenging task because these compounds are involved in a number of biochemical, physiological or pathological processes. Since the pK_{a1} of the acids is in the range of from 2.8 to 4.3 and the pK_{a2} of the second carboxyl group is from 5.4 to 5.7, both carboxyl groups are deprotonated at the neutral pH of the buffer. Owing to the high negative electrophoretic mobility of analytes the application of the negative voltage with changing the detection to anodic side is more preferable. As a rule, cationic surfactants are used to reduce or even reverse the direction of the EOF and achieve separation. As shown in Fig. 2A, the separation of the dicarboxylic acids

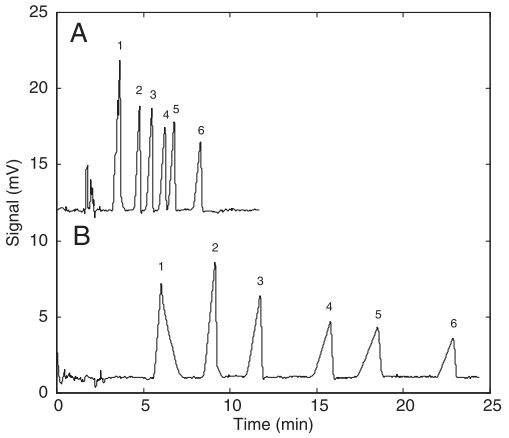

Figure 2. Separation of dicarboxylic acids in PEEK (A) and fused-silica (B) capillaries. Experimental conditions: capillary 75 μm id × 46 cm × 26 cm, 15 mM MES/His buffer pH 6.1, separation voltage +19 kV. Mixture of compounds at a concentration of 50 μM was injected electrokinetically for 3 s at +19 kV from the cathodic side. Peak identification: 1 – malonic acid, 2 – succinic acid, 3 – glutaric acid, 4 – phthalic acid, 5 – adipic acid, 6 – pimelic acid.

mixture in the PEEK capillary was achieved without any prior surface coating. The baseline resolution between six analytes (namely malonic, succinic, glutaric, phthalic, adipic and pimelic acids) was carried out using 15 mM MES/His buffer (pH 6.1). PEEK capillary parameters were 75 μm id × 46 cm × 26 cm, an applied voltage of +19 kV with anodic detection and sample electrokinetic injection at +19 kV for 3 s from the cathodic side. The concentration of each dicarboxylic acid in the sample mixture was 50 μM. The separation took place fairly fast and the experiment was accomplished in less than 8 min. The reproducibility of the migration time and peak area was estimated to be 5.5–7.7% and 4.1–6.4%, respectively. The electropherogram of the separation of dicarboxylic acids in the fused-silica capillary performed at the same electrophoretic conditions is shown in Fig. 2B. As seen, the time of analysis is noticeably longer and the peak shapes are broadened. The main explanation for this phenomenon could be the difference in EOF velocity between both the capillaries. Having two negatively charged groups, dicarboxylic acids could overcome the slow EOF in the PEEK capillary and move in the opposite direction toward the anode. The movement against the EOF contributes to the broadening of the sample zone that results in the distortion of the peak shape and the decrease in the separation efficiency (*N* values for the PEEK capillary are in the range of from 9013 to 20 183 plates/m). In the case of the fused-silica capillary, the EOF offers too strong resistance to the movement of sample zones leading to a three-fold increase in analysis time and a dramatic reduction in the efficiency ($N = 2824$–13 107 plates/m).

3.3 Humic acids

The separation of humic acids is difficult to carry out. The mixtures contain macromolecules having various functional groups, such as carboxylic, hyroxy, and phenyl, phenolic and quinolic groups. Capillary electrophoresis offers a unique possibility for analyzing humic substances and for obtaining information about their structure and properties. The pattern of humic substances mainly depends on sample structure, buffer composition and other experimental conditions. In the case of the fused-silica capillary, humic acid samples afford characteristic regions in the electropherogram: a spike (low molecular compounds) and a wide "humic hump" (sometimes with poorly defined shoulders) from heterogeneous matter with poorly defined structure [31–33]. However, analysis of humic substances in the bare fused-silica capillary is complicated due to the adsorption of the sample on the capillary wall. Figure 3A shows the electrophoretic pattern of humic acid using 15 mM MES buffer at low pH. The electropherograms obtained had hardly defined pattern and probably required greater sample concentration or modification of separation condition. In contrast, a representative electropherogram of humic acids using PEEK capillary is depicted in Fig. 3B. As in the case of fused-silica capillary, electrophoretic analysis was accomplished in the PEEK capillary using 15 mM MES (pH 4.5) as background electrolyte, separation voltage +19 kV and sample hydrodynamic injection at 10 cm height for 10 s. Humic acid samples afford a well-defined "humic hump" with some characteristic regions, which indicates the presence of several groups of compounds migrating at different electrophoretic velocities. Unlike fused silica, the main fractions were recognizable and reproducible (Fig. 4). The pattern of the humic sample in the PEEK capillary was sufficiently reproducible from run-to-run with an RSD value of peak as 5.4%. The experiments were performed at a traditional positive polarity without any invention of the complex buffer composition or modification of the capillary wall. On the other hand, using a low sample concentration (0.1 g/L) and the obtained stable and repeatable results could indicate overcoming of humic acids adsorption on the capillary wall in the case of the PEEK capillary. As a result, such undesirable factors as non-reproducible peaks, long retention time and the need for a relatively high sample concentration were eliminated. The results obtained may be used to investigate differences or similarities between humic samples of different origins, as well as to study the degradation processes.

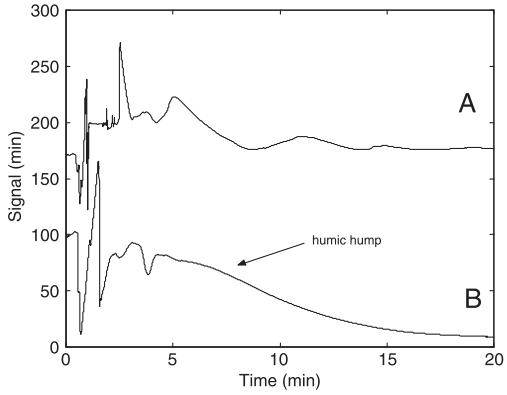

Figure 3. Electropherograms of humic acids in fused-silica (A) and PEEK (B) capillaries. Experimental conditions: capillary 75 μm id × 45 cm × 20 cm, 15 mM MES buffer pH 4.5, separation voltage of +19 kV, hydrodynamic injection at 10 cm height for 10 s, sample concentration 0.1 mg/mL.

3.4 Proteins and peptides

Peptides and proteins are one of the biological substances that are the most difficult to investigate by CE. However, the necessity to analyze them is beyond any doubt. The analysis of a complex biological mixture consisting of anionic and cationic proteins (cytochrome c, myoglobin, ribonuclease A, β-lactoglobulin A+B, α-lactalbumin) with different p*I* (4.5–10.6) and molecular weight (12.4–24 kDa) was carried out in a single run using the PEEK capillary. In

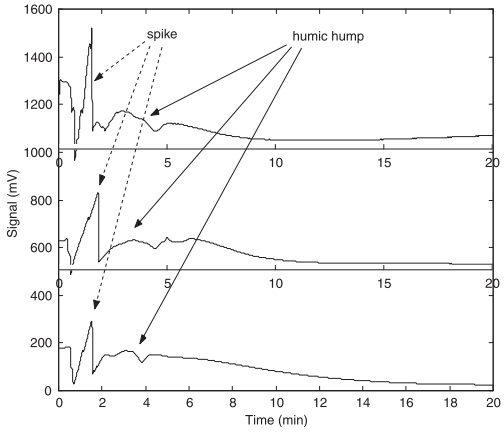

Figure 4. Reproducibility of the humic acid pattern. All experimental conditions are as in Figure 3.

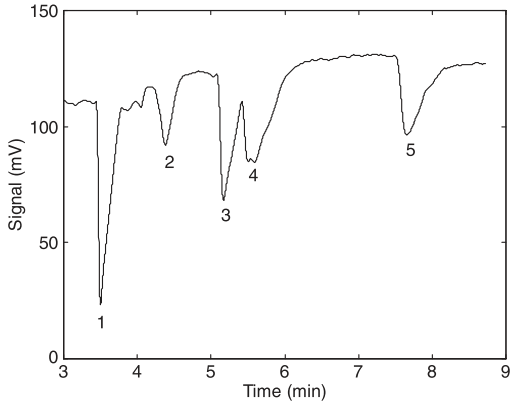

Figure 5. Electrophoretic separation of the proteins. Experimental conditions: PEEK capillary 75 μm id × 55 cm × 30 cm, 20 mM benzoic acid buffer pH 3.1, separation voltage +19 kV, electrokinetic injection 10 s at +19 kV, concentration of proteins in the sample 0.5 mg/mL. Peak identification: 1 – cytochrome c, 2 – myoglobin, 3 – ribonuclease A, 4 – β-lactoglobulin A/B, 5 – α-lactalbumin.

practice, the manipulation of the pH of the buffer is a powerful tool used to control the charge on the analyte and, hence, the resolution between the neighboring peaks in an electropherogram. At high pH, the protein is typically negatively charged and, furthermore, the possible adsorption will be minimized as a result of the charge repulsion effect. Twenty millimolar Tris (pH = 8.9) and 100 mM ammoniun hydroxide (pH = 9.8) were chosen and tested as separation buffers due to their suitability for C^4D. However, the analyses were not successful. The poor results may be accounted for the too weak EOF in the PEEK capillary which is unable to push the negatively charged high molecular analytes to the cathode. To overcome the problem, the possibility of carrying out sepration in the PEEK capillary at low pH was examined. As a result, all proteins having a positive charge migrate to the cathode due to their own electrophoretic mobility. After a number of experiments, 20 mM benzoic acid as background electrolyte (pH 3.1), separation voltage of +19 kV, a 10 s electrokinetic injection at +19 kV s proved to be the optimal conditions to achieve a resolution between all proteins in the PEEK capillary (75 μm id × 55 cm × 30 cm). A typical electropherogram is presented in Fig. 5. As the pH of the buffer is well below the p*I* of basic proteins the latter are eluted before acidic proteins due to the high positive charge. The longer migration time of acidic proteins is due to the smaller positive charge present at this pH (close to the p*I* of the protein). The analysis was fast (about 9 min), the results were obtained at a concentration of 0.5 mg/mL. The calculated values of theoretical plates indicated the relatively low effectiveness (7840–13 693 plates/m) of the protein separation. The relative standard deviation of the migration time and peak area was close to 10%. The shift in the migration time and decrease in efficiency are indicative of the reversible adsorption of the protein onto the capillary wall. Beside the electrostatic interactions, the adsorption–desorption processes may also affect the band width of the protein zone as migration occurs. Especially at extreme pH, the hydrophobic areas of protein could be exposed, resulting in the hydrophobic adsorption. Owing to the hydrophobic nature of the material of the PEEK capillary, an undesirable interaction between the capillary wall and protein may take place, leading to the tailing of peaks. So, the untreated PEEK capillary did not prove suitable to be used for the direct analysis of complex protein mixtures and further studies will be required. This method may be used only as preliminary express analysis, but to obtain accurate quantitative results, the processing of the capillary wall surface is necessary. Attempts to perform experiments in the fused-silica capillary at the same conditions were not successful.

Additionally, the separation of a model peptide mixture (Gly-Tyr, Val-Tyr-Val, Met-enkephalin, Leu-enkephalin, angiotensin II) was carried out in the PEEK capillary without any modification of the buffer composition or capillary wall coating. Figure 6A illustrates the electrophoretic separation of peptides at a concentration of 0.25 mg/mL using optimized conditions: PEEK capillary 75 μm id × 64 cm × 44 cm, 20 mM benzoic acid buffer (pH 3.1), separation voltage +19 kV, electrokinetic injection at +19 kV for 10 s. Peptides were successfully separated within 12 min according to an increase in their molecular weight. A minimum resolution was achieved between Met-enkephalin and Leu-enkephalin, which differ in only one amino acid residue. The calculated number of theoretical plates for five peptides resulted up to 75 000 plates/m. Moreover, the RSD values of migration time of peptides were in the range of 2.9–3.8% ($n = 5$). The electropherogram of the standard peptide mixture obtained in the fused-silica capillary at the same conditions is also shown in Fig. 6B. The separation of

all compounds was also achieved even in a shorter time. The electropherograms obtained show that the two patterns differ, which is also indicative of the difference in the mechanisms of separation taking place in the fused-silica and PEEK capillaries.

For each compound analyzed such important parameters as the effectiveness of separation and reproducibility of migration time and peak area were calculated and are presented in Table 1. The higher RSD values may result from the use of electrolytes with low buffering capacity due to the exploitation of the conductivity detection, the movement of the sample against the EOF and the use of extreme pH conditions. The quantitative response of CE-C^4D in the PEEK capillary to compounds was also investigated and summarized in Table 1. The detection limits were determined as concentrations giving a ratio of mean peak area (A_{mean}) to areas standard deviation (sA) equal to three (*i.e.* $S/N = 3*A_{mean}/sA$). The obtained values for non-stacking sample injection are relatively high, being in the micromolar range. Apparently, sensitivity could be increased in the case of method development for specific purpose.

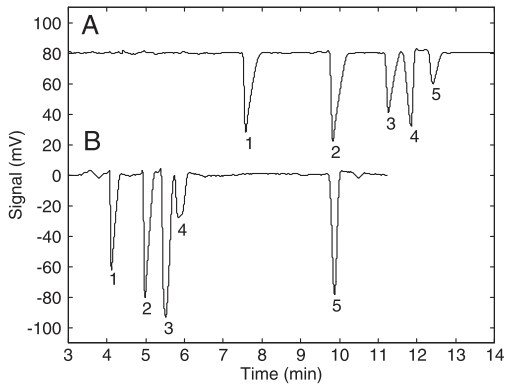

Figure 6. Electrophoretic separation of the peptides mixture in (A) PEEK and (B) fused-silica capillaries. Experimental conditions: capillary 75 μm id × 64 cm × 44 cm, 20 mM benzoic acid buffer pH 3.1, separation voltage +19 kV, electrokinetic injection 5 s at +19 kV, concentration of each peptide in the sample 0.25 mg/mL. Peak identification: 1 – Gly-Tyr, 2 – Val-Tyr-Val, 3 – Met-enkephalin, 4 – Leu-enkephalin, 5 – angiotensin II.

4 Concluding remarks

In this work the possibility of using PEEK capillaries as an alternative to conventional fused-silica capillaries has been shown. Its resolving power, in combination with the high efficiencies achieved, makes the PEEK capillary useful for the analysis of complex biological samples. The investigations demonstrated the possibility of application of this polymer capillary for the separation of various classes of compounds. Low EOF, reduced sample adsorption on the capillary wall, simple experimental conditions and good reproducibility of the results are the main presented advantages of using the PEEK capillary. Moreover, PEEK capillaries may be used in combination with contactless conductivity detector without any need for the detection window and low optical transperancy is no longer a hindrance. The only noted drawback is a mechanical softness of PEEK material, which caused some technical

Table 1. Performance of the PEEK capillary in the analysis of compounds under study

Analyte	RSD (%)[a]		Efficiency (plate/m)[b]	LOD (μM)[c]
	Migration time	Peak area		
Malonic acid	5.7	4.1	9013	6.2
Succinic acid	5.5	6.4	10 155	9.6
Glutaric acid	6.3	4.6	13 624	6.9
Phthalic acid	7.1	5.1	20 183	7.7
Adipic acid	7.7	3.9	15 783	5.9
Pimelic acid	7.4	4.7	18 410	7.1
Gly-Tyr	5.5	3.6	20 236	11.4
Val-Tyr-Val	5.8	5.9	28 116	18.7
Met-enkephalin	5.5	4.9	45 182	15.4
Leu-enkephalin	6.0	3.3	75 427	10.3
Angiotensin II	5.5	3.1	55 995	9.7
Cytochrome c	5.8	7.4	9084	9.0
Ribonuclease A	6.1	6.7	13 693	8.2
Myoglobin	6.8	7.0	11 907	8.6
α-Lactalbumin	7.3	10.3	7840	12.5
β-Lactoglobulin A/B	7.5	10.4	10 929	12.6

a) Values were obtained from an average at least of three independent runs.
b) The number of theoretical plates was calculated as $N = 5.54[t_i/(\text{area}_i/\text{peak height}_i)]^2$ and re-calculated to capillary effective length.
c) Limits of detection (calculated as a triple ratio of mean peak area to areas standard deviation).

problems, particularly during the installation of capillary into the detection cell. Furthermore, using the C^4D the determination of biomolecules of different sizes from small organic acids to proteins was simple and there was no need for derivatization procedure. The PEEK capillary is a potential candidate for developing portable, point-of-care devices to analyze *in situ* complex biological samples.

The authors have declared no conflict of interest.

5 References

[1] Heiger, D. N., *High Performance Capillary Electrophoresis – An Introduction*, Hewlett Packard, France 1992, 136p.

[2] Melanson, J. E., Baryla, N. E., Lucy, C. A., *Trends Anal. Chem.* 2001, *20*, 365–374.

[3] Hsieh,Y.-Y., Lin, Y.-H., Yang S.Jr., Wei, G.-T. *et al.*, *J. Chromatogr. A* 2002, *952*, 255–266.

[4] Sebastiano, R., Citterio, A., Righetti, P. G., Simo-Alfonso, E., Ramis-Ramos, G., *J. Chromatogr. A* 2004, *1053*, 235–239.

[5] Hardenborg, E., Zuberovic, A., Ullsten, S., Söderberg, L. *et al.*, *J. Chromatogr. A* 2003, *1003*, 217–221.

[6] Denton, K. A., Harris, R. J., *J. Chromatogr. A* 1995, *705*, 335–341.

[7] Shen, Y., Smith, R., *Electrophoresis* 2002, *23*, 3106–3124.

[8] Verzola, B., Gelfi, C., Righetti, P. G., *J. Chromatogr. A* 2000, *874*, 293–303.

[9] Puerta, A., Axén, J., Söderberg, L., Bergquist, J., *J. Chromatogr. B* 2006, *838*, 113–121.

[10] Mikšik, I., Deyl, Z., *J. Chromatogr. A* 1999, *852*, 325–336.

[11] Hult, E. L., Emmer, A., Roeraade, J., *J. Chromatogr. A* 1997, *757*, 255–262.

[12] Corradini, D., *J. Chromatogr. B* 1997, *699*, 221–256.

[13] Catai, J. R., Tervahauta, H. A., de Jong, G. J., Somsen, G. W., *J. Chromatogr. A*, *1083*, 185–192.

[14] Becker, H., Locascio, L. E., *Talanta* 2002, *56*, 267–287.

[15] Shadpour, H., Musyimi, H., Chen, J., Soper, S. A., *J. Chromatogr. A* 2006, *1111*, 238–251.

[16] Kim, M. S., Cho, II. S., Lee, K. N., Kim, Y. K., *Sensors Actuators B* 2005, *107*, 818–824.

[17] Benavente, F., Sanz-Nebot, V., Barbosa, J., van der Heijden, R. *et al.*, *Electrophoresis* 2007, *28*, 944–949.

[18] Schneider, P. J., Engelhardt, H., *J. Chromatogr. A* 1998, *802*, 17–22.

[19] Tanyanyiwa, J., Leuthardt, S., Hauser, P. C., *J. Chromatogr. A* 2002, *978*, 205–211.

[20] Macka, M., Yang, W. C., Zakaria, P., Shitangkoon, A. *et al.*, *J. Chromatogr. A* 2004, *1039*, 193–199.

[21] Rohlicek, V., Deyl, Z., Mikšik, I., *J. Chromatogr. A* 1994, *662*, 369–373.

[22] Bayer, H., Engelhardt, H., *J. Microcol. Sep.* 1996, *8*(7), 479–484.

[23] Chiang, S. C. C., Li, S. F. Y., *Biomed. Chromatogr.* 1997, *11*, 366–370.

[24] Miller, L., Juza, M., *J. Chromatogr. A* 2005, *1094*, 165–168.

[25] Zemann, A. J., *Trends Anal. Chem.* 2001, *20*, 346–354.

[26] Zemann, A. J., *Electrophoresis* 2003, *24*, 2125–2137.

[27] Šolinova, V., Kašicka, V., *J. Sep. Sci.* 2006, *29*, 1743–1762.

[28] Govindaraju, K., Govindaraju, V., Eidelman, D. H., *J. Chromatogr. B* 2003, *788*, 369–376.

[29] Tanyanyiwa, J., Schweizer, K., Hauser, P. C., *Electrophoresis* 2003, *24*, 2119–2124.

[30] Abad-Villar, E. M., Tanyanyiwa, J., Fernández-Abedul, M. T., Costa-Garcia, A., Hauser, P. C., *Anal. Chem.* 2004, *76*, 1282–1288.

[31] Pokorná, L., Pacheco, M. L., Havel, J., *J. Chromatogr. A* 2000, *895*, 345–350.

[32] Weinberger, R., *Am Lab* 2007, *39*, 34–35.

[33] Cavani, L., Ciavatta, C., Trubetskaya, O. E., Reznikova, O. I. *et al.*, *J. Chromatogr. A* 2003, *983*, 263–270.

PUBLICATION II

K. Helmja, M. Borissova, T. Knjazeva, M. Jaanus, U. Muinasmaa, M. Kaljurand, M. Vaher. Fraction collection in capillary electrophoresis for various stand-alone mass spectrometers. J. Chromatogr. A 2009, 1216, 3666-3673.

Journal of Chromatography A, 1216 (2009) 3666–3673

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Fraction collection in capillary electrophoresis for various stand-alone mass spectrometers

Kati Helmja[a], Maria Borissova[a], Tatjana Knjazeva[a], Martin Jaanus[b], Urmas Muinasmaa[a], Mihkel Kaljurand[a], Merike Vaher[a,*]

[a] Department of Chemistry, Faculty of Science, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia
[b] Department of Computer Control, Faculty of Information Technology, Tallinn University of Technology, Ehitajate tee 5, 19086 Tallinn, Estonia

ARTICLE INFO

Article history:
Available online 27 December 2008

Keywords:
Capillary electrophoresis
Fraction collection
Mass spectrometry
Bioactive compounds

ABSTRACT

A procedure for collecting fractions during capillary electrophoresis for their analysis using various stand-alone instruments is described. The results of a systematic study of the optimization and application of capillary electrophoresis (CE) in conjunction with a reverse-phase high-performance liquid chromatography electrospray ionization quadrupole time of flight-tandem mass spectrometry (RP-HPLC-ESI-Q-TOF-MS/MS) and inductively-coupled mass spectrometry (ICP-MS) to the analysis of the seed extract of the Japanese Pagoda Tree (*Sophora japonica*) are presented. The off-line coupling of CE to the matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) for the proteins mixture was applied. The cathode end of the capillary was placed inside a stainless steel needle using a coaxial liquid-sheath-flow configuration. The optimization of experimental parameters resulted in an efficient methodology for MS analysis of fractions. Several components contained in the extract of *S. japonica* were identified, some not previously known. It was demonstrated that low sensitivity, which is a real problem in off-line CE–MS analysis, could be tolerated because of a more flexible optimization of the CE separation conditions and the choice of independent stand-alone instruments for analysis of separated fractions. The estimated limit of detection for CE-RP-HPLC-ESI-Q-TOF-MS was 50 μM of polyphenols and for CE-ICP-MS, 1–100 μg/l.

1. Introduction

Capillary electrophoresis (CE) has, with different modifications such as capillary zone electrophoresis (CZE), micellar electrokinetic electrochromatography (MEKC) and capillary isoelectric focusing (CIEF), gained in popularity as a tool for different types of analysis due to advantages such as speed of analysis, low amount of sample/buffer and high efficiency. However, a few disadvantages, such as detection capability, need to be improved. The coupling of CE to MS has been demonstrated to be a sensitive and powerful method of detection. Additionally, CE–MS allows identification of unknown compounds and characterization of analytes according to structure. CE–MS has been successfully applied to the analysis of a wide range of analytes (small molecules, metabolites, amino acids, etc.) [1–4]. Several modifications have been made in coupling on-line CE to MS. The weakest point has been the interface between CE and MS because of the necessity of applying a relevant voltage over the separation capillary together with the correct voltage needed for performing ESI. This requires a careful consideration of the current values of ESI and CE. Nowadays various interfaces have been proposed [1–5], but electrospray ionization (ESI) has mainly been used. However, the successful on-line coupling of CE to MS requires a volatile buffer. Because of non-volatility and the risk of MS source contamination, the most common buffers used in CE, such as borate and phosphate, as well as additives such as sodium dodecyl sulfate (SDS), are not suitable for coupling CE to ESI-MS [3]. Buffers such as acetic acid, ammonium carbonate and ammonium acetate have proven to be appropriate for use in MS [2,3]. However, these buffers may not allow satisfactory separation of analytes of interest. Hence, a solution could be to apply an off-line CE with fraction collection to set up a combination of CE with MS. The off-line CE–MS is considered a convenient approach due to its flexibility towards system optimization since the experimental conditions for the CE and MS instruments can be optimized separately [6]. Additionally, it enables using different types of ion sources for MS, such as matrix-assisted laser desorption/ionization (MALDI), inductively-coupled plasma (ICP) and electrospray.

Fraction collection by CE is difficult to accomplish in yields sufficient for a further study of analytes by various stand-alone spectroscopic techniques. Because CE sample injection volumes are very low, the concentrations of separated analytes are also very low. On the other hand, CE fraction collection is desirable

* Corresponding author.
E-mail address: merike@chemnet.ee (M. Vaher).

0021-9673/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2008.12.061

because CE often enables separation of analytes that cannot be resolved by other techniques. Moreover, CE offers unique operating modes for experimenting with nanoliter volumes of samples such as electrophoretically mediated microanalysis or affinity electrophoresis or monitoring of the reaction kinetics, in which case characterization of individual sample bands is highly desirable. A characteristic of such cases is the fact that CE peak identity can be determined at the very beginning of the study. Once the peak identity has been established, the subsequent study can rely on the measurement of peak areas only, taking advantage of the recognition of the CE pattern. This approach makes the development of fraction collection systems that can rely on off-line use of expensive service instruments, especially mass spectrometers, to be shared among different research groups, reasonable.

The first collection of fractions in capillary electrophoresis was demonstrated by Hjerten and Zhu in 1985 on nucleosides, pH markers and IEF ampholytes [7]. Fraction collection is a popular approach for coupling CE with MALDI because it is difficult to perform on-line CE-MALDI-MS since MALDI usually has to be carried out in vacuum and proteins have to be co-crystallized with the matrix on the surface before analysis. Therefore, off-line coupling systems, which allow automated protein spotting, were introduced [8,9]. The sheath-flow fraction collection set-up has proven to be the simplest device to interface CE with MALDI. A droplet of the CE effluent is mixed with the matrix solution at the capillary tip and deposited on the MALDI target. After all fractions have been collected on the MALDI plate, the droplet is transferred into the mass spectrometer and analyzed. Various off-line interfaces have been proposed, mainly differing in the way the CE effluent is deposited on the MALDI plate [10–12].

Off-line coupling of CIEF to MALDI-TOF MS is a promising tool for the analysis of proteins. The technique has been successfully applied to the analysis of model protein systems [13–15]. Crowley and Hayes were the first to analyze real samples (human blood serum) by using CIEF–MALDI-MS [16]. Off-line coupling of CE with a microwave-assisted acid hydrolysis/MALDI-MS has been developed for protein identification and characterization [17]. The CE collection of fractions into PCR tubes containing 5 μl of a dilute separation buffer was performed electrokinetically. Recently, Lechner et al. described a combination of CIEF with MALDI-MS using a sheath liquid-assisted automatic sample deposition device (Dionex "Probot") [18]. Silvertand et al. studied the effect of different concentrations and brands of carrier ampholytes, detergents and polymers on CIEF repeatability and separation efficiency, as well as on the main detection signals (UV and MS) [19]. Zhou and Johnston reported on the application of a two-dimensional approach, using CIEF and reversed-phase liquid chromatography (RPLC) combined with ESI-MS for the analysis of intact proteins [20,21]. Applied to a yeast sample, the method enabled detection of up to 500 proteins and polypeptides in the mass range from 3 to 30 kDa and with a p*I* between 3 and 11. A transient CITP-CZE-based multidimensional separation apparatus was constructed by Balgley et al. [22–24]. The cathode end of the capillary was housed inside a stainless steel needle using a coaxial liquid-sheath-flow configuration. A sheath liquid composed of 0.1 M acetic acid was delivered at a flow rate of 1 μl/min using a syringe pump. The stacked and resolved peptides in the CITP-CZE capillary were sequentially fractionated and loaded into individual wells on a moving microtiter plate. To couple the transient CITP-CZE with nano-RPLC, the peptides collected in individual wells were sequentially injected into individual trap columns packed with porous C18 reversed-phase particles. Each peptide fraction was subsequently analyzed by nano-RPLC-ESI-MS.

The 2-D CE – (off-line) – CE equipment was designed by Santos et al. by modifying a commercially available CE–MS system [25]. The fractions were collected in microvials containing 1 μl electrophoretic buffer. Zamfir et al. suggested the use of off-line high-performance capillary electrophoresis in connection with nanospray ESI Q-TOF for the identification of complex carbohydrates of biological origin [26]. In another study the performance of CE and an off-line CE-ESI-Q-TOF-MS approach was explored for screening a complex ganglioside mixture from bovine brain [27]. Also, fraction collection systems have been reported for microchip CE [28,29].

In this paper an attempt has been made to apply a generic fraction collection approach to several other spectroscopic methods. Moreover, the fractions can be further treated by chemical or biological means. It has been demonstrated that besides using MALDI-TOF-MS, the fractions can be analyzed by a microcolumn HPLC-ESI-Q-TOF-MS and ICP-MS as well. In this work a novel approach is that the fractions are subjected to the analysis by a microcolumn HPLC-ESI-MS implementing HPLC as a second dimension. In a few attempts to combine CE with HPLC the latter is considered a first dimension just because of the detection limits problems: HPLC can accept several orders of more sample than CE. It is demonstrated here that the coupling can be done the other way around: CE acting as a first dimension by scarifying resolution in CE dimension. A motivation for such an approach could be the fact that HPLC–MS is a well-established technique (why not to take advantage of that) whereas CE–MS is still in its infancy. A few attempts have been made to build-up on-line ICP-MS. Due to the high cost of such experiment – both the instrument and consumables are expensive – this approach is not widely spread and only a few laboratories have used it. The off-line coupling seems to be more relevant since it gives access to a stand-alone instrument and consumes much less instrument time and consumables. For MALDI the generic approach is rather a standard one and many of the existing fraction collectors for MALDI are not instrument specific. In this work MALDI has been used just to emphasize the advantages of the generic approach.

Using the CE instrument as a fraction collector, the concentration of the eluted sample is low, being diluted by electrolyte in the sample reservoirs to provide volumes of a few nanoliters diluted into tens of microliters. The lack of sensitivity is a specific and well-known drawback of this approach; however, in this paper we would like to demonstrate that it can be tolerated by using various stand-alone instruments to detect analyte molecules in the CE-collected fractions. To our knowledge this is the first time a generic fraction collector has been proposed, the performance of which is not tailored to a specific instrument. While the majority of publications on fraction collection deals only with proteins and peptides, in this work we attempted the separation and analysis of small molecules found in the methanol extract of a herbal sample, the seed of *Sophora japonica*, using off-line coupling of CE with a micro-column RP-HPLC-ESI-Q-TOF-MS or ICP-MS. *S. japonica* was chosen (as a model object of analysis) just because the electropherograms of its methanol extract contains a few well separated and intense peaks. Also, its extract is of interest of traditional medicine in the Far East [30–33]. We propose a simple fraction collector based on an overflow of the capillary end by a sheath liquid forced by gravity, with collection of liquid droplets into PCR microvials located on the *xy*-stage. This fraction collection system can collect samples for various spectrometric instruments. Although the fraction collection system dilutes the CE zone by about two orders of magnitude, the analysis of the methanol extract of *S. japonica* demonstrates that the characterization of the main components of the extract by microcolumn RP-HPLC-ESI-Q-TOF-MS and ICP-MS is possible.

2. Experimental

2.1. Chemicals

All reagents were of analytical grade and were used as received. Flavone, naringin, rutin, quercitrin hydrate, sinapic acid, sodium hydroxide, sodium tetraborate, trifluoroacetic acid, acetonitrile and acetic acid were obtained from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Methanol was purchased from Rathburn Chemicals Ltd (Walkerburn, Scotland). The stock solutions of standards (5 mM) were prepared in methanol and stored at +4 °C in the dark. Standard proteins cytochrome *c*, human serum albumin, trypsinogen, lysozyme, ribonuclease A, α-lactalbumin and human hemoglobin were purchased from Sigma–Aldrich (Steinheim, Germany). The stock solutions of proteins (1 mM) were prepared in Milli-Q water. Nitric acid was "Suprapure" grade (Merck, Darmstadt, Germany). The multi-element standard solutions of 10 mg/l (High-Purity Standards, Charleston, USA) were used to prepare calibration solutions.

For preparation of all solutions and samples, high purity deionized water (18.2 MΩ/cm) from the Milli-Q system (Millipore, Milford, USA) was used.

2.2. Sample preparation

The seeds of *S. japonica* were purchased from a local specialist store supplying various traditional medicinal products. The extract of *S. japonica* was prepared as follows. The seeds were dried at room temperature and milled into powder. For the extraction of phenolic components 0.5 g of the sample was weighed and 5 ml of the extraction solvent (methanol) was added. The sample was left at room temperature for 60 min and followed by ultrasonic extraction for 20 min. The extraction procedure was performed in triplicate. The extracts were collected and concentrated to a 2 ml volume by a rotary evaporator (Laborota 4000/4001, Heidolph Instruments GMBH, Germany). The extract was filtered through a 0.45 μm filter and stored at +4 °C in the dark.

2.3. Instrumentation and conditions

2.3.1. Capillary electrophoretic fraction collection

The CE apparatus was constructed in-house using a 1000R high-voltage power supply (Spellman High-Voltage Electronics, Plainview, NY). For analysis of flavonoids and the herbal extract an uncoated silica capillary (Agilent Technologies) with a total length of 70 cm (an effective length of 57 cm, 100 μm i.d./365 μm o.d.) was used. Prior to use the capillary was rinsed with a 0.1 M NaOH solution for 3 min and with the separation buffer, 25 mM sodium tetraborate, at pH 9.3 for 3 min. The extract was diluted three times before introduction into the CE capillary. The injection of the sample was performed either hydrodynamically (Δh = 20 cm) or electrokinetically for 15 s. A positive electrical voltage of 15 kV was applied to the inlet reservoir for separation. An UV absorbance detector at 210 nm was placed at 13 cm from the cathode end of the capillary. The detector signal was transferred to a personal computer via a self-made 16-bit analogue-to-digital-converter. The data were handled by MATLAB Version 6.0 Release 12 (The MathWorks, Inc., Natick, MA, USA).

The separation of protein mixture was performed in a polyvinyl alcohol (PVA) coated silica capillary (Agilent Technologies) with a total length of 52 cm (an effective length of 39 cm, 50 μm i.d./365 μm o.d.). The capillary was preconditioned with 10 mM phosphoric acid, Milli-Q water and buffer solution each for 3 min. In the separation and fraction collection study a 250 mM acetic acid (pH 3) was used; the voltage applied was +20 kV. The mixture of seven standard proteins (10 μM of each) was injected electrokinetically (at +20 kV for 8 s).

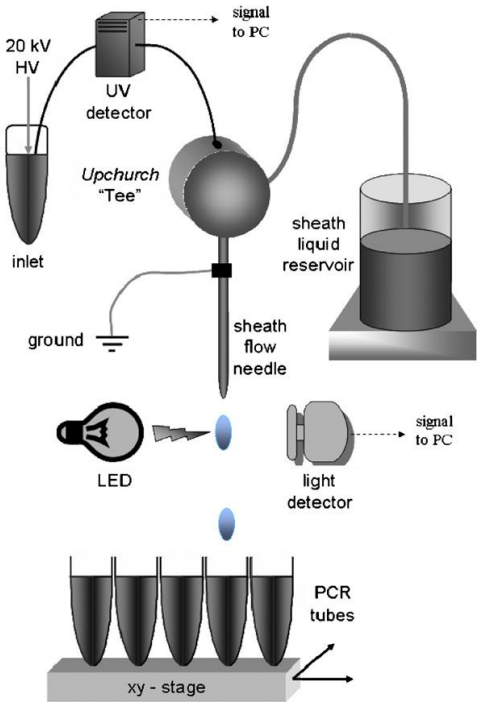

Fig. 1. A schematics of the sample collector.

The fraction collector is depicted in Fig. 1. The cathode end of the capillary was housed inside a stainless steel needle using a coaxial liquid-sheath-flow configuration. A sheath liquid composed of 250 mM or of 1 M acetic acid was delivered by gravity (the vessel rose above the stainless steel needle endpoint by approximately 10 cm) at a flow rate of 2.5 μl/s. Concentration of acetic acid was chosen to match of the ionic strength of the separation buffer. The sheath liquid left the stainless steel needle as a sequence of 12 μl or 18.5 μl droplets depending of the capillary diameter. The resolved analytes in the CE capillary were sequentially fractionated into droplets that were collected into individual 250 μl PCR tubes located in the wells on a moving *xy*-stage on a microtiter plate. CE separation and fractionation were complete in less than 15 min.

The droplets were counted by a light detector with a built-in light emitting diode (LED) driver circuit (Optoswitch S4282-51, Hamamatsu). This only responds to the luminous flux of the connected LED, because the flux to the LED is modulated and a signal processor synchronizes the sensitivity of the light detector to the modulation. The unit can tolerate high background light levels. The output level is TTL/CMOS-compatible and readable using a parallel computer interface.

2.3.2. RP-micro-HPLC and mass spectrometry

To couple CE with micro-RPLC, the analytes collected in individual wells were sequentially injected and subsequently analyzed by micro-RPLC consisting of a P680 chromatographic pump (Dionex, Sunnyvale, CA) equipped with a degasser (Dionex Corporation, Sunnyvale, USA). The pump was fitted with a nanoflow splitter and a SB-C18 reverse-phase microcolumn (0.5 mm × 75 mm, 3.5 μm, Agilent), the outlet of which was connected to an ESI fused-

Table 1
The operating parameters of an Agilent ICP-MS 7500a.

Parameter	Value
Nebulizer	Babington-type
Spray chamber	Glass, double pass
Sampler cone	Ni, orifice diameter 1.0 mm
Skimmer cone	Ni, orifice diameter 0.4 mm
Spray chamber temp	2 °C
Plasma gas flow rate	15.0 l/min
Carrier gas flow rate	1.27 l/min
RF Power	1350 W
Sample uptake rate	0.21 ml/min
Sampling depth	8 mm
Points/mass	3
Analysis time/mass	0.1–0.60 s
Oxide ratio (156/140)	<0.5%
Double charged (70/140)	<1.5%
Sensitivity	^{89}Y >15,000 cps/ppb

silica capillary (50 μm i.d. × 165 μm o.d.). The samples (3 μl) were injected into the HPLC system without any pre-treatment. The mobile phase consisted of 0.1% trifluoroacetic acid (solvent A) and acetonitrile. The elution was allowed to run from 90% to 0% solvent A for 40 min. The system was reconditioned with the initial gradient for 10 min. A flow rate of 15 μl/min was achieved by splitting the initial flow rate.

A Q-Star Elite mass spectrometer (Applied Biosystems, Germany) equipped with an ESI source was used for all Q-TOF experiments. Mass spectra were obtained in a full scan mode (200–1000 amu). The instrument was operated in a positive ion mode under the following conditions: needle voltage, 4.5 kV; sheath gas (N_2), 80 psi; auxiliary gas (N_2), 29 psi. Collision energy for fragmentation was optimized by Q-Star Elite software depending on MS–MS ion intensity 50 V. The MS–MS spectrum was obtained for the most intense four peaks in each survey scan and the extracted ion-current (EIC) strategies were used to analyze each fraction of the extract.

2.3.3. ICP-MS and MALDI-TOF-MS

The determination of 31 elements, viz. Ag, Al, As, Ba, Be, Bi, Ca, Cd, Co, Cr, Cu, Eu, Fe, Ho, K, La, Li, Mg, Mn, Mo, Ni, P, Pb, S, Sb, Sr, Th, Tl, U, V, Zn, in the *S. japonica* extract was carried out using an inductively-coupled plasma mass spectrometer Agilent ICP-MS 7500a (Agilent Technologies Inc., USA). The minimum sample amount required for ICP-MS analysis, 400 μl of 1% nitric acid solution, was added to all fractions. The sample uptake rate was minimized and the carrier gas flow rate was optimized to allow analysis of small volume samples (>400 μl) with sufficient sensitivity. The configuration and operating conditions for the instrument are listed in Table 1. The external calibration was performed by using multi-element standard solutions. The internal standards (Ge, Y, Rh, Re, each 50 μg/l) were continuously added to all samples and solutions by using the second channel of a peristaltic pump.

An Autoflex III MALDI-TOF mass spectrometer (Brucker Daltonics, MA, USA) equipped with a nitrogen laser (337 nm) and operated in the linear mode applying the accelerating voltage of 20 kV for protein measurements was used. The laser power was kept constant, and the other parameters were adapted automatically by the instrument's software. The spectrum were obtained by accumulating 500 laser shots in the positive mode of operation. All sample preparations were performed on a stainless steel plate using a dried-droplet technique. For that, each collected fraction (~12 μl) and 2 μl of the matrix solution were mixed and briefly vortexed. Then 1.5 μl of the solution was spotted directly into the MALDI plate and allowed to air-dry at room temperature. A 10 mg/ml solution of sinapic acid in 50% (v/v) acetonitrile in Milli-Q water and 0.1% (v/v) TFA was used as the matrix for protein mass-spectral analysis. In order to lower the limit of detection the procedure of sample deposition into the MALDI plate was repeated three times: the second sample layer was deposited on the top of the first layer, allowed to dry and then the whole procedure was repeated once again.

3. Results and discussion

In general, it is acknowledged that the background electrolyte required by CE for the separation of certain analytes may not be suitable if CE is coupled to MS in an on-line mode. The off-line mode can circumvent this hurdle by using a sheath liquid which is compatible with MS. In this work, acetic acid was used as a sheath liquid. To ensure that acetic acid does not interfere with the separation of analytes in the capillary, the speed of migration of acetic acid ions must be smaller than the electro-osmotic flow (EOF) on the anionic side. The experimental mobilities of acetic acid ions and the EOF were compared. The mobilities obtained were 6.4×10^{-8} m^2 s^{-1} V^{-1} and -2.6×10^{-8} m^2 s^{-1} V^{-1} for the EOF and an acetic acid ion, respectively, meaning that sheath-liquid ions would probably not migrate into the CE capillary and would not interfere with the separation of analytes.

The fraction time was roughly estimated based on the migration time of the peak observed at the UV detector. Assuming that the mobility of the analyte is constant during electrophoresis, the time at which the analyte band reaches the capillary outlet (t_o) is computed from the migration time corresponding to the top of the analyte peak as its zone passes through the detector window (t_d). The computation is performed as follows: $t_o = (l_o/l_d)t_d$, where l_o is the full length of the capillary and l_d the effective length of the capillary to the detector.

3.1. CE-RP-micro-HPLC-ESI-MS

Validation of the fraction collection system was performed using a mixture of the standard solution consisting of flavone, naringin, rutin and quercitrin. The electropherogram of these four polyphenol standards is shown in Fig. 2A. Flavone migrates with EOF, which explains the shape of the peak. Below the electropherogram a "comb-like" signal from the droplet counter is seen. The time interval between two peaks of this signal corresponds to the time during which a droplet is formed. Because the sheath liquid is delivered by gravity the interval between two consecutive droplet falls-off increases slightly as the level of the sheath liquid vessel decreases (the sheath liquid delivery by a syringe pump is straightforward if such an increase cannot be tolerated). The arrows with numbers indicate fractions for which a HPLC–MS signal was detected (note apparent shift compared to the CE signal due to the detector position at 13 cm from the cathode end of the capillary). The corresponding mass spectra of the four standards are shown in Fig. 2B. The rest of the fractions appeared to be empty within the detection limit of the fraction collection method used and are not shown. Total ion chromatograms (TIC) are not given since they contained only one peak and are of no interest. The mass spectrum of fraction #1 contains a molecular ion peak of flavone ($(M_{\text{flavone}} + 1)/z = 223$) and that of fraction #4 contains a molecular ion peak of rutin ($(M_{\text{rutin}} + 1)/z = 611$. The mass spectrum of fraction #2 contains two peaks. A peak at $(M_{\text{naringin}} + 1)/z = 581$ corresponds to a molecular ion of naringin and a peak at $m/z = 273$ is apparently due to the partial fragmentation of naringin at the ionization source (due to the breaking of a weak glycoside bond): This was also confirmed by MS/MS fragmentation of the molecular ion peak at $m/z = 581$ which resulted, two peaks $m/z = 581$ and $m/z = 273$. Similar reasoning can be applied to the mass spectrum of fragment #3. A peak at $(M_{\text{quercitrin}} + 1)/z = 449$ is a molecular

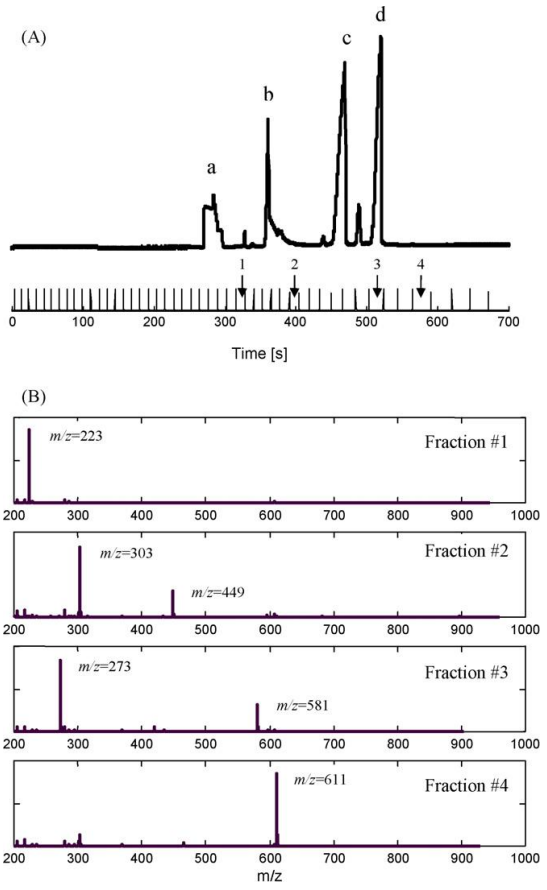

Fig. 2. CE-ESI-MS analysis of standard mixture of the flavonoids. (A) Electropherogram of the solution of standards (1 mM) with an electrical impulse (stripes) of each exiting droplet in the anionic side of the capillary: a – flavone; b – naringin; c – rutin; d – quercitrin. (B) Mass spectra of fractions. The CE separation conditions: separation buffer 25 mM sodium tetraborate (pH 9.3), sheath liquid 1 M acetic acid; the effective length of the capillary 57 cm, applied voltage +15 kV, UV detection at 210 nm, injections were performed hydrodynamically over 15 s.

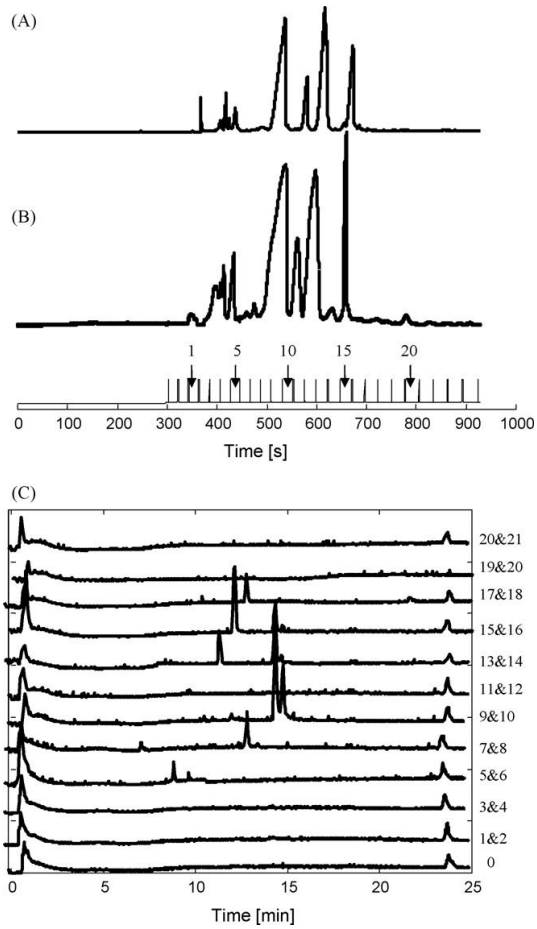

Fig. 3. Analysis of the extract of *S. japonica*. (A) electropherogram of the extract; (B) the electropherogram of the concentrated extract; (C) corresponding TICs of the fractions. The CE separation conditions were the same as in Fig. 2.

ion peak of quercitrin and at $m/z = 303$ there is another fragment.

The detection limits of concentration of the initial sample (i.e. the one introduced into the capillary) for fraction collection were estimated as follows. First, the sample dilution that takes place during the migration of analytes along the capillary column is not taken into account since we assumed that the whole band of the analyte is trapped in one fraction. Similarly, because the MS software is able to integrate over the whole TIC peak the sample dilution due to the HPLC process can be ignored. Fractionation by a sheath liquid is responsible for most of the dilution. Assuming that in the present case a typical volume of the CE band of 0.105 μl (calculated from the apparent peak base and the migration time of the quercitrin peak in Fig. 2A) is diluted by approximately 18.5 μl droplet, a dilution factor of 176 times is obtained. The concentration of each analyte in the sample extract of standards was 100 μM each and they were detected as ESI-TIC peaks with a signal-to-noise ratio S/N = 6 (for naringin S/N = 4). A dilution factor of 176 times gives a detected concentration of 0.3 μM. Thus, 50 μM (assuming S/N = 3 at TIC) seems to be the limit of detection for the CE-RP-HPLC-ESI-TOF-MS combination. Performance data for TIC peak area reproducibility is 4%.

An electropherogram *of S. japonica* extract is shown in Fig. 3A demonstrating a nice separation. Unfortunately, by HPLC–MS analysis of this sample a detectable signal was obtained only for very few fractions. By overloading the capillary (the extracts were concentrated four times) the analysis of more fractions became possible. One can easily recognize and make a one-to-one peak correspondence between Fig. 3A and B. Thus, if the identification of a peak is an issue, then the scarifying separation quality for increasing the detection limit could be justified. A droplet counter signal with droplet numbers is shown below the electropherogram. The corresponding TICs are shown in Fig. 3C. For an off-line recording of the mass spectra two droplets were merged together to reduce the number of fractions analyzed by MS. A peak at a retention time of 24 min appears in all chromatograms and is due to the fraction collection system used (probably a compound leached out by acetic acid from the PCR tube). Most fractions (composed of two droplets) contain just one peak. However, a fraction from the droplets #(11&12) contains two peaks. The same two compounds can also be found in the next fraction. Thus, in fact, the fraction collection system performs a two-dimensional separation. The mass-spectral

Table 2
The compounds identified in the extract of *S. japonica* by ESI-Q-TOF.

Fraction #	Retention time (min)	*m/z* data		Compound
		M + 1	MS/MS	
7&8	9.0	595	595 → 433, 271	Genistein glucoside
	9.5	741	741 → 595, 433, 271	Genistein rutinoside glucoside
9&10	6.2	[a]		Unknown
	13.5	433	433 → 271	Genistin
	13.7	463	463 → 301, 286	Methoxykaempferol glucoside
11&12	9.9	757	757 → 595, 433, 287	Kaempferol diglucoside rhamnoside
13&14	10.8	[a]		Unknown
	14.5	865	865 → 433 → 271	Genistin-dimer
	14.7	579	579 → 433, 271	Sophorabioside
15&16	12.3	757	757 → 595, 449, 287	Kaempferol rutinoside glucoside
	14.5	433	865 → 433 → 271	Genistin-dimer
	14.7	579	579 → 433, 271	Sophorabioside
17&18	12.0	611	611 → 287	Kaempferol sophoroside
	14.2	595	595 → 449, 287	Cyanidin rutinoside
	14.6	625	625 → 479, 317, 302	Methylquercetin rutinoside
19&20	10.6	773	773 → 611, 449, 303	Quercetin diglucoside rhamnoside
	13.0	611	611 → 449, 465, 303	Rutin
	22.3	271	271 → 243, 215, 153	Genistein

[a] Mass not detected.

data relating to the peaks are given in Table 2. The main components in the extract were genistin, sophorabioside and genistein glucosides. These results are supported by published data [30,31]. It follows from the mass-spectral data that several CE peaks can be identified using the fraction collection system tested. While the presence of some compounds in the *S. japonica* extract (to the best of the authors' knowledge) has been reported previously, compounds such as metoxykaempferol glucoside, kaempferol rutinoside glucoside, cyanidin rutinoside, methylquercetin rutinoside and quercetin diglucoside rhamnoside were found in the extract for the first time.

Determination of the exact concentrations of the compounds identified was not possible since the ionization efficiency of different analytes could have varied and most standards of the compounds identified were not available. To determine the approximate concentration of compounds in the initial 15 ml extract of *S. japonica* seed powder (0.5 g) various factors should be taken into account. Evaporating the initial 15 ml extract by vacuum evaporation to 2 ml increases the concentration 7.5 times. The extract was diluted three times before CE analysis (to maintain CE resolution at an acceptable level) and 176 times by the droplet, which results in an overall dilution factor of the initial extract of about 70 times in the droplet. However, merging two droplets dilutes the sample twice more and the injection of only 10 μl of the 37 μl fraction into the HPLC column (to provide aliquots for estimation of reproducibility) reduces the sample amount for MS analysis a further seven times. Thus, the initial extract is diluted 500 times and this number, together with the LOD values obtained for polyphenol standards (0.3 μM), can be used to estimate the concentration of the main components of the extract, which could be about 150 μM.

The fractions could have been introduced into the MS directly. However, discussing the advantages of the microcolumn HPLC the column provides a needed orthogonal separation dimension if CE alone cannot separate analytes. This can be well seen in Fig. 3C in case of fractions 13&14 and several others. Moreover, the HPLC column acted as a concentrator medium for the analytes diluted in the fraction volume. We were unable to see the predominant mass spectra of the analytes if the fractions were introduced into the MS directly. Although it is not directly implemented here, the HPLC column could be used for separating analytes from unwanted sheath liquid components when they are not MS compatible.

3.2. CE-ICP-MS analysis of the Sophora japonica extract

First, the non-fractionated methanol extract of *S. japonica* seed powder (0.25 g/ml) was subjected to the ICP-MS analysis following the analysis of the fractions collected. The concentration of most of the tested elements was below detection limits. Only those metals were targeted to the CE-ICP-MS analysis which signal was above the detection limit in the extract. Besides macroelements (K, P, S and Ca; concentration ranging between 1 and 50 mg/l) the extract was found to contain significant concentrations of Cu, Fe, Mg, Mn, Ni and Zn (concentrations ranging between 10 and 200 μg/l). The distribution of some metals over different fractions (fractogram) is represented in Fig. 4 (the droplet numbers correspond to those in Fig. 3). The results of analysis of Fe and Ni are not given because the results may have been influenced by the material leaching from the electrode. The peaks in the fractograms of Mg (50 μg/l), Mn

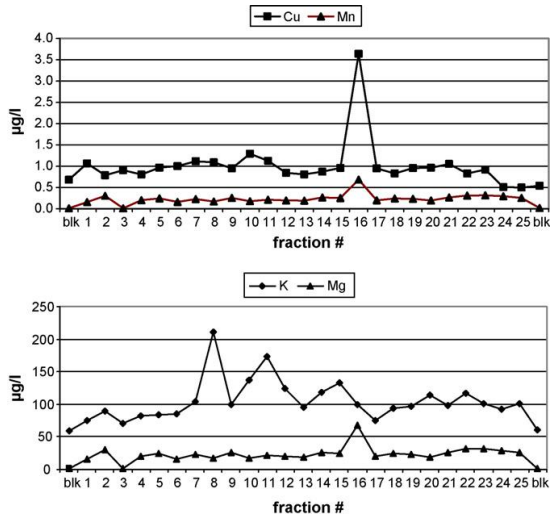

Fig. 4. The results of the ICP-MS analysis of the *S. japonica* extract. The "blk" refers to the ICP-MS signal from Milli-Q water. Note that the *y*-axis of the Cu signal is shifted by 1 μg/l units and that of the K signal by 100 μg/l units.

Table 3
Reproducibility statistics for CE-MALDI-TOF measurements.

Protein	RSD (%)[a]		Molecular weight (Da)		
	Migration time	Peak area	Literature[b]	Experimental[a]	SD[a]
Cytochrome *c*	1.8	2.5	12300	12225.75	35.38
Lysozyme	2.3	3.0	14300	14301.60	44.07
Human serum albumin	1.6	3.3	66500	66616.99	157.85
Ribonuclease A	2.5	1.4	13700	13695.88	12.11
Trypsinogen	1.9	3.2	24000	23985.63	66.91
α-Lactalbumin	1.4	2.5	14200	14179.32	34.34
Human hemoglobin	1.9	3.5	64000	15142.14	15.23
				15884.89	12.15

[a] RSD, SD and experimental average are based on 5 replicate measurements.
[b] Literature molecular weight from reagent's producer datasheets.

(0.5 μg/l) and Cu (2.5 μg/l) can be associated with particular peaks in the electropherogram and might represent some type of association of metals with the other components of the extract. The particular type of association was not investigated. On the other hand, the fact that none of the components identified by ESI-Q-TOF were metallo-complexes indicates that the latter are contained in *S. japonica* at very low concentrations and could not be detected. If metals are present in the extract in an ionic form, then they possibly eluted with the injection peak during HPLC analysis and could not be detected by MS either. K was found in many droplets. A number of various other metals were represented at the trace level. Elements P, S and Zn were not found on fractograms while Ca was found in all fractions. We have no explanation for the latter two observations.

3.3. CE-MALDI-TOF-MS analysis of the protein standards solution

An electropherogram of the mixture of protein standards is presented in Fig. 5A. The droplet counter signal and fraction numbering through one are shown at the bottom of the electropherogram. The mass spectra of the collected fractions are shown in Fig. 5B, with the exception of that the fraction appeared to be empty or the amount of the protein was negligible. In the spectrum recorded, the main ion peaks $[M+H]^+$ were detected. A variation in signal intensity was also observed between the different fractions (the mass spectrum of

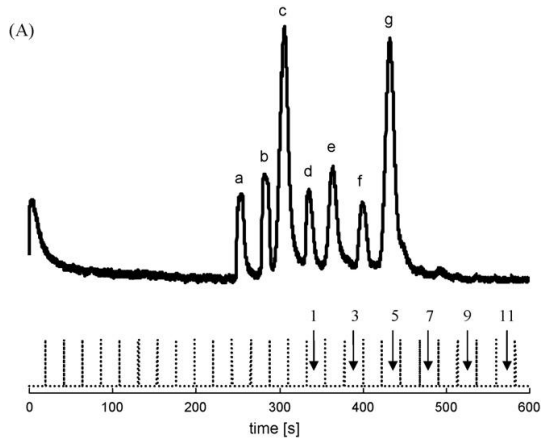

Fig. 5. Analysis of the protein standards. (A) Electropherogram with an electrical impulse. Separated proteins: a – cytochrome *c*; b – lysozyme; c – human serum albumin; d – ribonuclease A; e – trypsinogen; f – α-lactalbumin; and g – human hemoglobin. (B) Mass spectra of the collected fractions. The CE separation conditions were as: PVA coated capillary 52 cm/39 cm, i.d. 50 μm; buffer and sheath liquid 250 mM acetic acid; voltage 20 kV, positive polarity; detection 210 nm; injection 8 s at +20 kV; sample concentration 10 μM of each.

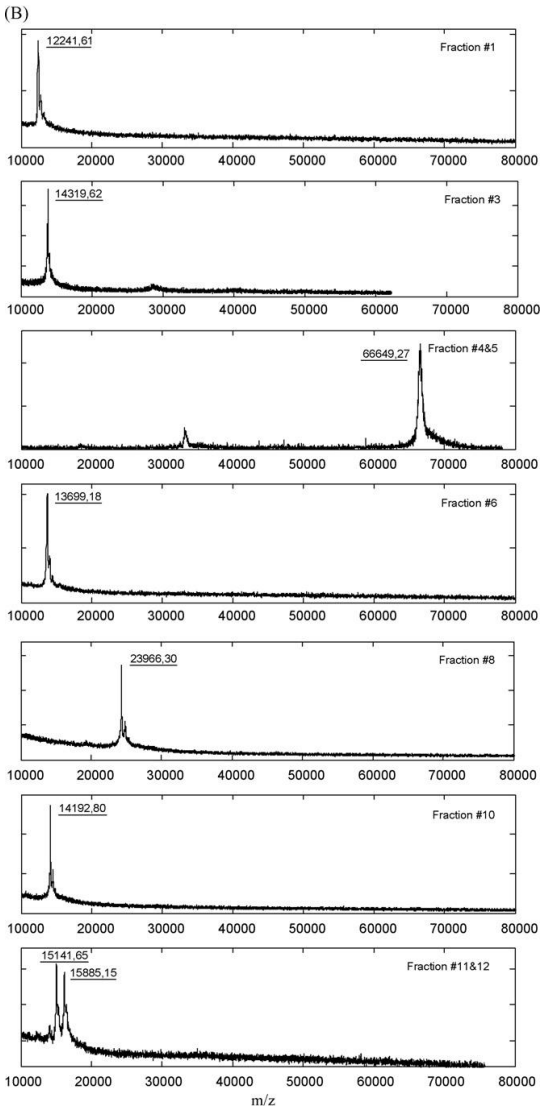

Fig. 5. (*Continued*)

low molecular weight proteins result in the higher signal intensity). In general, the major peaks observed in each mass spectrum correspond to the proteins expected according to the CE signal in Fig. 5A. The mass spectrum of fraction #1 contains only a single peak of cytochrome *c*. In fraction #3 the signal from lysozyme is observed. The next two mass spectra are indicative of the presence of human serum albumin in fractions #4 and #5, respectively. The peak in the mass spectrum of fraction #6 is identified as ribonuclease A. In fraction #7 negligible quantities of ribonuclease A and trypsinogen are observed, but the main signal of trypsinogen is detected in the subsequent fraction #8. The mass spectrum of fraction #10 contains a peak of α-lactalbumin. The hemoglobin is identified in fractions #11 and #12 containing two neightbouring paks. This fact could be explained by a tetrameric structure of hemoglobin, consisting of two α and two β subunits which are bound noncovalently and dissociate during the ionization step. The data on the accuracy of the reproducibility of the protein electrophoretic separation and MALDI-TOF-MS measurements are presented in Table 3.

4. Conclusion

In summary, the off-line CE-ESI-MS approach used to investigate the herbal extract is advantageous in view of compatibility of CE with ESI-Q-TOF or ICP-MS conditions. The off-line coupling of CE to MALDI-TOF could be useful for the analysis of complex proteomic samples due to requirement prior fractionation for simplification of MS profiles and also for the removal of species which could interfere with the MALDI analysis.

Also, as CE is completely autonomous, the collection of fractions in the form of droplets enables all problems related to the electrical coupling between CE and MS to be avoided. A droplet of the sheath liquid acts as a container, the size of which is ergonomically compatible with human operator capabilities, which in turn enables complex manual manipulations (addition of reactants, concentration, dilution and physical processing) of the fractions to be performed if needed for further study of fractions by various methods. No interruption of the separation voltage during the electropherogram run was needed, unlike in the case of several other fraction collectors. It was possible to precisely correlate the time of falling of the droplet with the detector signal event.

The reduced sensitivity, which is a major drawback of this approach, can be explained by the fraction collection principle of the off-line approach, which currently can be partially compensated for by the use of the high sensitivity and dynamic range Q-TOF or ICP analyzer in MS detection. The necessity of decreasing detection limits could lead to an increase in the amount of injected sample, which will inevitably lead to the loss of resolution on the electropherogram. However, this can be compensated for by the use of the RP micro-column separation in the second dimension. Besides low sensitivity and resolution, the third disadvantage of fraction collection in CE is the heavy manual work that is required. Despite this, manual fraction collection is still frequently used by many research groups. However, fraction collection could be performed by a robotic spotter [18], while the MS analysis is possible using an autosampler. In general, the type of fraction collection developed in this work could be envisioned as supporting a project consisting of the study of a high number of samples with similar composition. Once the main composition of an average sample has been established, the effect of various factors on sample composition can be further studied without involving MS. A good example of such an approach is the study of the antioxidative properties of herbal extracts [34–36], because CE performs exceptionally well in the separation of herbal extracts, food and other agricultural products.

Acknowledgement

The support of the Estonian Science Fund (Grant no. ETF7818) is acknowledged.

References

[1] C.W. Huck, G. Stecher, H. Scherz, G. Bonn, Electrophoresis 26 (2005) 1319.
[2] C. Simó, C. Barbas, A. Cifuentes, Electrophoresis 26 (2005) 1306.
[3] C.W. Klampfl, Electrophoresis 27 (2006) 3.
[4] A. Gaspar, M. Englmann, A. Fekete, M. Harir, P. Schmitt-Kopplin, Electrophoresis 29 (2008) 66.
[5] E. Gelpí, J. Mass Spectrom. 37 (2002) 241.
[6] L. Bindila, R. Almeida, A. Sterling, M. Allen, P. Peter-Katalinic, A. Zamfir, J. Mass Spectrom. 39 (2004) 1190.
[7] S. Hjerten, M.-D. Zhu, J. Chromatogr. A 327 (1985) 157.
[8] K.L. Walker, R.W. Chiu, C.A. Monnig, C.L. Wilkins, Anal. Chem. 67 (1995) 4197.
[9] J.A. Castoro, R.W. Chiu, C.A. Monnig, C.L. Wilkins, J. Am. Chem. Soc. 114 (1992) 7571.
[10] H. Stutz, Electrophoresis 26 (2005) 1254.
[11] C.W. Huck, R. Bakry, L.A. Huber, G.K. Bonn, Electrophoresis 27 (2006) 2063.
[12] S.I. Snovida, V.C. Chen, O. Krokhin, H. Perreault, Anal. Chem. 78 (2006) 6556.
[13] F. Foret, O. Muller, J. Thorne, W. Gotzinger, B.L. Karger, J. Chromatogr. A 716 (1995) 157.
[14] M. Minarik, F. Foret, F.B.L. Karger, Electrophoresis 21 (2000) 247.
[15] A. Chartogne, M. Gaspari, S. Jespersen, B. Buscher, E. Verheij, R. Van der Heijden, U. Tjaden, J. Van der Greef, Rapid Commun. Mass Spectrom. 16 (2006) 201.
[16] T.A. Crowley, M.A. Hayes, Proteomics 5 (2005) 3798.
[17] M.M. Yassine, N. Guo, H. Zhong, L. Li, C.A. Lucy, Anal. Chim. Acta 597 (2007) 41.
[18] M. Lechner, A. Seifner, A.M. Rizzi, Electrophoresis 29 (2008) 1974.
[19] L.H.H. Silvertand, J.S. Toraño, G.J. de Jong, W.P. van Bennekom, Electrophoresis 29 (2008) 1985.
[20] F. Zhou, M.V. Johnston, Electrophoresis 26 (2005) 1383.
[21] F. Zhou, M.V. Johnston, Anal. Chem. 76 (2004) 2734.
[22] X. Fang, L. Yang, W. Wang, T. Song, C.S. Lee, D.L. DeVoe, B.M. Balgley, Anal. Chem. 79 (2007) 5785.
[23] W.J. Wang, T. Guo, T. Song, C.S. Lee, B.M. Balgley, Protemics 7 (2007) 1178.
[24] T. Guo, C.S. Lee, W.J. Wang, D.L. DeVoe, B.M. Balgley, Electrophoresis 27 (2006) 3523.
[25] B. Santos, B.M. Simonet, Á. Ríos, M. Valcárcel, Electrophoresis 28 (2007) 1345.
[26] A. Zamfir, S. Konig, J. Althoff, J. Peter-Katalinic, J. Chromatogr. A 895 (2000) 291.
[27] A. Zamfir, Z. Vukelic, J. Peter-Katalinic, Electrophoresis 23 (2002) 2894.
[28] C.S. Effenhauser, A. Manz, H.M. Widmer, Anal. Chem. 67 (1995) 2284.
[29] D.R. Zalewski, S. Schlautmann, R.B.M. Schasfoort, H.J.G.E. Gardeniers, Lab Chip 8 (2008) 801.
[30] R. Liu, Y. Qi, A. Sun, H. Xie, J. Sep. Sci. 30 (2007) 1870.
[31] J.-H. Wang, F.-C. Lou, Y.-L. Wang, Y.-P. Tang, Phytochemistry 63 (2003) 463.
[32] Y. Qi, A. Sun, R. Liu, Z. Meng, H. Xie, J. Chromatogr. A 1140 (2007) 219.
[33] R. Gevrenova, G. Kitanov, D. Ilieva, Pharm. Biol. 45 (2007) 14.
[34] M. Vaher, S. Ehala, M. Kaljurand, Electrophoresis 26 (2005) 990.
[35] K. Helmja, M. Vaher, T. Püssa, K. Kamsol, A. Orav, M. Kaljurand, J. Chromatogr. A 1155 (2007) 222.
[36] J. Gorbatsova, T. Lougas, R. Vokk, M. Kaljurand, Electrophoresis 28 (2007) 4136.

PUBLICATION III

T. Knjazeva, M. Kaljurand. Capillary electrophoresis frontal analysis for the study of flavonoid interactions with human serum albumin. Anal. Bioanal. Chem. 2010, 397, 2211-2219.

Anal Bioanal Chem
DOI 10.1007/s00216-010-3726-4

ORIGINAL PAPER

Capillary electrophoresis frontal analysis for the study of flavonoid interactions with human serum albumin

Tatjana Knjazeva · Mihkel Kaljurand

Received: 29 January 2010 / Revised: 19 March 2010 / Accepted: 6 April 2010

Abstract Capillary electrophoresis based on the principles of frontal analysis (CE-FA) was used to characterize the binding of flavonoids to human serum albumin (HSA) at near-physiological conditions: 67 mM phosphate buffer (pH 7.4), temperature 36.5 °C. The studied flavonoids (flavone, rutin, quercitrin) displayed moderate affinities toward the human serum albumin with binding constants in the range 10^3–10^4 M^{-1}. The binding of the flavonoids to the protein noticeably depended on their lipophilicity and decreased in the case of glycosylation. The corresponding thermodynamic parameters characterized the acting forces between the HSA and flavonoids as mainly hydrophobic forces and electrostatic interactions. Based on the results of the displacement experiments, the binding of the flavonoids took place at site I of the HSA molecule. The results demonstrated by CE-FA were similar to those obtained by fluorescence spectroscopy. The developed method proved to be a reliable alternative to conventional methods, providing a lot of useful parameters for characterization of ligand–protein interactions.

Keywords Capillary electrophoresis · Frontal analysis · Binding constant · Flavonoids · Human serum albumin

Presented at the 6th Instrumental Methods of Analysis Conference (IMA 2009) held in Athens, Greece, 4–8 October 2009.

T. Knjazeva (✉) · M. Kaljurand
Department of Chemistry, Faculty of Science,
Tallinn University of Technology,
Akadeemia tee 15,
12618 Tallinn, Estonia
e-mail: tatjana.knjazeva@yahoo.com

Introduction

Comprehensive studies on the nature of interactions between small molecules and proteins are of fundamental importance for biomedical and pharmaceutical sciences. The overall distribution, duration and intensity of physiological actions, as well as the metabolism and elimination, of many drugs in the human body are correlated with affinities towards plasma proteins. In this context, the characterization of binding properties has found a broad application in new drug development and the investigation of biologically active compounds.

Several analytical methods have been developed for the characterization of small ligand interactions with proteins, including equilibrium dialysis, ultrafiltration, ultracentrifugation [1], fluorescence spectroscopy [2], nuclear magnetic resonance [3] and surface plasmon resonance [4]. Capillary electrophoresis in the format of frontal analysis (CE-FA) is a relatively new method of investigating binding processes. Based on the principles of zone electrophoresis and using commercial CE instrumentation, this technique affords the efficient and accurate determination of binding parameters. In frontal analysis, the premixed protein–ligand sample is injected as a large plug and separated during electrophoresis into the free ligand and protein/protein–ligand complex zones due to the differences in mobility. Nevertheless, the equilibrium is maintained where the zones overlap. The key advantages of CE-FA are the near-physiological conditions and rapidity of analysis, ease of automatization, small sample consumption, robustness and accuracy of results, which are all extremely useful for the accurate and rapid characterization of binding processes [5–8].

Human serum albumin (HSA) is one of the most abundant proteins in blood plasma at a physiological concentration of about 35–50 g l^{-1}. Consisting of 585 amino acids, the single-

Published online: 27 April 2010

chain unglycosylated polypeptide is assembled as a heart-shaped molecule with three homologous domains (I–III), each divided into subdomains A and B. Human serum albumin has a number of physiological functions involving the binding and transport of physiologically important molecules [9, 10]. The binding capability is largely due to the existence of two major hydrophobic binding sites commonly named as Sudlow's site I and site II, located within particular cavities in subdomains IIA and IIIA, respectively. Site I ligands are mainly large heterocyclic negatively charged compounds and site II ligands are generally small aromatic carboxylic acids [11–13]. The majority of publications are devoted to interactions of HSA with a variety of compounds covering a wide range of properties, such as cationic drugs [6], anti-cancer metallodrugs [14], antibiotics [15, 16] and organic acids [17].

Among the variety of pharmaceutical compounds, natural products have always attracted considerable interest because of their potential beneficial effects on human health. Flavonoids, aromatic secondary metabolites of plants, are an important class of natural products identified in a broad range of fruits, vegetables, herbs and some beverages. In plants, the majority of flavonoids are found as glycosides with different sugar groups linked to the hydroxyl groups by glycosidic linkages [18, 19]. The biological activities of flavonoids were first reported in 1936 by Rusznyàk and Szent-Gÿorgyi, who found that red pepper and citrus extracts were effective in the prevention of capillary bleeding and fragility [20]. Subsequently, numerous studies have demonstrated scientific evidence of antiviral, anti-tumour, anti-allergic, anti-inflammatory and anti-mutagenic activities, and hepato- and gastro-protective properties [18, 19, 21, 22]. These positive biomedical effects are mostly attributed to the potential of flavonoids to act as free radical scavengers and their antioxidant activity has also been widely studied [23–25]. Despite the emerging evidence of the relationship between a flavonoid-rich diet and health benefits, demonstrated association remains unclear. For this reason, a detailed study of interactions between dietary flavonoids and serum albumin could be helpful for a better understanding of their transportation and absorption mechanisms.

In the present work, capillary electrophoresis frontal analysis was used for characterization of interactions between human serum albumin and three structurally different flavonoids: flavone as the basic form, the monoglycoside quercitrin and diglycoside rutin (Fig. 1). Besides determination of the binding constants, the thermodynamic parameters of binding processes were characterized and the specific binding site for flavonoids to HSA was identified. This study significantly contributes to the application of CE-FA as a worthy alternative to traditional methods, allowing the determination of a variety of binding parameters and the characterization of physiological behaviours of bioactive compounds.

Materials and methods

Chemicals and solutions

All reagents were obtained as analytical grade. Albumin from human serum (essentially fatty acid free), rutin hydrate, quercitrin hydrate, flavone, warfarin, ibuprofen, sodium dihydrogen phosphate dihydrate, sodium hydroxide and sodium dodecyl sulfate (SDS) were obtained from Sigma-Aldrich (Steinheim, Germany). Methanol was purchased from Rathburn Chemicals Ltd (Walkerburn, Scotland).

For all CE-FA experiments, phosphate buffer solution at a concentration 67 mM and pH 7.4 was used. The buffer was prepared by dissolving an appropriate amount of sodium dihydrogen phosphate dihydrate in Milli-Q water and adjusting the pH by the addition of 1 M NaOH. Prior to use, the buffer solution was filtered through a 0.45-μm nitrocellulose Millipore filter. The stock solutions of rutin, quercitrin and flavone were prepared at a concentration of 3 mM in methanol and stored at +4 °C in the dark. The working solution of HSA at a concentration 10 mg/ml was prepared in 67 mM phosphate buffer and stored in the refrigerator prior to use. The stock solutions of warfarin and ibuprofen (3 mM) were prepared in methanol. All further dilutions of stock solutions and mixture preparations were made in separation buffer in order to prevent disequilibrium changes in the reaction mixture during the electrophoretic analysis and to avoid system peak appearance.

Instrumentation

The CE-FA experiments were performed on an Agilent CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array UV/Vis detection system. The uncoated fused silica capillaries with total length 38.5 cm (30 cm to the detector), 50-μm i.d. and 375-μm o.d. were obtained from Agilent Technologies. Each new capillary was conditioned by rinsing with 1 M sodium hydroxide solution for 20 min, then with 0.1 M sodium hydroxide for 20 min and finally with Milli-Q water for 20 min. At the beginning of each day and between the runs, the capillary was conditioned by rinsing with 14 mg/ml SDS solution, 0.1 M NaOH, Milli-Q water and separation buffer at 50 mbar for 3 min for each solution. The capillary in the CE cartridge was thermostated at the appropriate temperature (36.5 or 25 °C). Before injection the samples were also thermostated by using a Techne DB-3A heater (Bibby Scientific Ltd, UK). Data were collected and analysed by

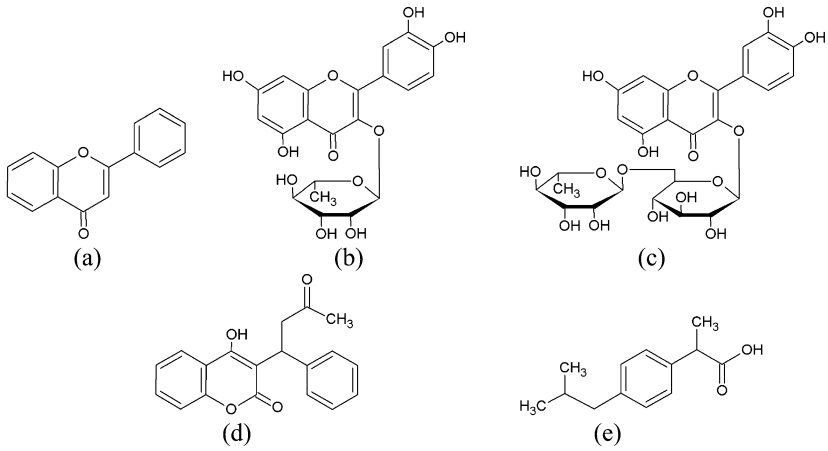

Fig. 1 Structural formulas of the analytes: **a** flavone, **b** quercitrin, **c** rutin, **d** warfarin and **e** ibuprofen

using ChemStation software (Agilent Technologies) and the Solver algorithm. The fluorescence measurements were carried out on an Hitachi F-7000 fluorescence spectrophotometer (Hitachi High Technologies Corporation, Japan) with a xenon lamp and quartz cell thermostated at the appropriate temperature. The log*P* values were estimated by using the miLogP software version 2.2. (Molinspiration Chemoinformatics, Slovakia) available as freeware [26]. The pH was measured by using a 744 pH Meter (Metrohm, Herisau, Switzerland). A Milli-Q water purification system (Millipore S.A., Molsheim, France) was used for producing deionized water of a high purity.

Procedure for CE-FA experiments

Study of flavonoid–HSA interactions

For CE-FA analysis, a series of mixtures was prepared in which the HSA concentration was kept constant (40 μM) and rutin, quercitrin or flavone solutions were added in different amounts to reach concentrations in the range of 50–500 μM. The samples were thoroughly mixed and equilibrated for 30 min at the appropriate temperature. Hydrodynamic injection at 50 mbar at the anodic end of the capillary was used to introduce sample and high voltage of +8 kV was applied for separation. Each flavonoid–HSA mixture was prepared and measured three times to ensure reproducibility of the results. The samples were thermostated and then analysed at two different temperatures, 36.5 and 25 °C.

Identification of binding site

The flavonoid and HSA solutions were thoroughly mixed in a 1:1 ratio and then the site marker (warfarin or ibuprofen) was added to the mixture. The concentrations of flavonoid, HSA and site marker were all kept at 100 μM. The CE-FA experimental conditions were the same as mentioned above.

Data processing and calculations

The concentration of flavonoid which remained unbound after incubation with HSA was estimated by measuring the height of the corresponding plateau peak on the electropherogram. For this purpose, a series of diluted solutions of rutin, quercitrin and flavone in the working concentration range were prepared and analysed under the same conditions as for the HSA–flavonoid mixtures. The amount of bound flavonoid $[F]_b$ was determined as the difference between the total $[F]_t$ and free $[F]_f$ flavonoid concentration:

$$[F]_b = [F]_t - [F]_f \tag{1}$$

The binding curves for each flavonoid–HSA system were constructed by plotting the number of bound ligand molecules per protein molecule (r) against the free flavonoid concentration. The binding constant (K) and the number of sites (n) were obtained by adjusting the experimental data using nonlinear regression according to the following equation [27]:

$$r = \frac{[F]_b}{[P]} = \frac{nK[F]_f}{1 + K[F]_f} \tag{2}$$

where $[F]_f$ and $[F]_b$ are the free and bound flavonoid concentrations and $[P]$ is the protein concentration.

The binding percentage of flavonoids towards HSA was evaluated by using Eq. 3:

$$B(\%) = \frac{[F]_b}{[F]_t}\ 100\% \tag{3}$$

Fluorescence quenching of HSA

A series of solutions was prepared in which concentrations of rutin varied from 2.5 to 40 μM and the amount of HSA was kept constant (1 μM). All of the samples were diluted in a separation phosphate buffer solution (67 mM, pH 7.4). The obtained mixtures were incubated at 36.5 °C for 0.5 h. The intensity of fluorescence was measured at 340 nm (exitation wavelength 280 nm) and corrected to the background fluorescence of buffer. The binding constant was estimated by plotting F_0/F versus $[R_t]F_0/(F_0-F)$ according to the common procedure [28] where F_0 and F are the fluorescence intensity of HSA in the absence and presence of rutin (R_t), respectively.

Results and discussion

Optimization of CE-FA conditions

An increased injection volume of sample and a difference between electrophoretic mobility of free ligand from the protein and the protein–ligand complex were the primary requirements for CE frontal analysis. Human serum albumin (p*I*=4.7) was mostly negatively charged in the physiological pH range and due to a high molecular mass it tended to migrate slowly towards the anode. Quercitrin and rutin were also slightly negatively charged due to the presence of partly dissociated phenolic−OH groups with a pK_a in the range 6.74–11.65 [29]. Flavone, having zero electrophoretic mobility, migrated with the electroosmotic flow under these conditions. Therefore, it was suggested that the flavonoid–HSA complex and HSA migrated as a single zone because a bound flavonoid does not notably change the charge and weight of the protein and its electrophoretic mobility. Flavonoid and flavonoid–HSA complex (co-migrated with free protein) were observed in the electropherogram as completely separated ordinary CE peaks when the injection time was short. In order to investigate plateau formation, different injection times (5–60 s) were examined with the same sample solution (150 μM rutin and 40 μM HSA). As can be seen in Fig. 2, a plateau region of free flavonoid started to appear with the 20-s injection but a more evident plateau was observed when the sample injection time was about 40 s. Moreover, with increasing injection time, the peaks became broadened and equilibrium was maintained in the overlapping zone. Thus, an injection time of 40 s at 50-mbar pressure was chosen for all further experiments.

Interactions of flavonoid with human serum albumin

The study of the interactions between human serum albumin and flavone, rutin and quercitrin was performed

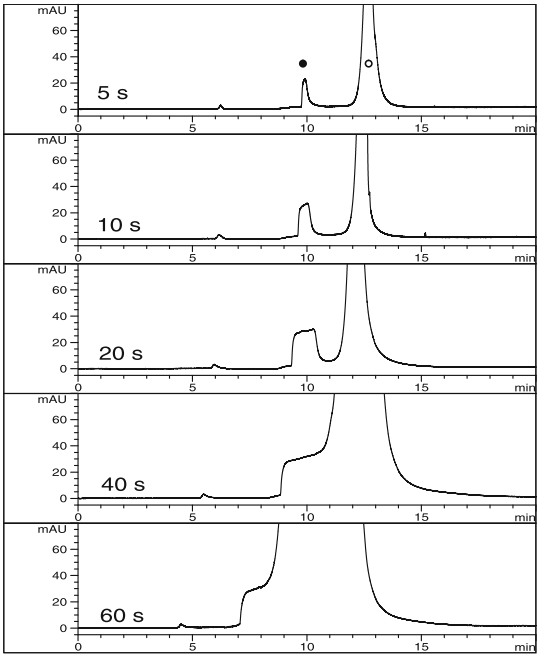

Fig. 2 Effect of injection time on plateau formation. Experimental conditions: 67 mM phosphate buffer (pH 7.4), capillary 50-μm i.d.× 37 cm×45 cm, applied voltage +8 kV, hydrodynamic injection at 50 mbar, UV detection at 210 nm; sample: 150 μM rutin (●) and 40 μM HSA (○)

under optimized conditions and according to above-mentioned CE-FA procedure. In the pre-equilibrated mixture some of the ligand is present in the free form whilst the rest is in a complex with the protein. The typical CE-FA electropherograms of standard flavone and the flavone–HSA mixture are illustrated in Fig. 3. As can be observed, the height of the plateau corresponding to the free flavone decreased in the presence of HSA, thereby proving the formation of the complex. Based on the experimental data, the binding curves of flavone, quercitrin and rutin were plotted as the number of bound ligand molecules per protein molecule (r) versus the free flavonoid concentration, as shown in Fig. 4. The binding constant (K) and the number of binding sites (n) obtained by the CE-FA method for the investigated flavonoids are summarized in Table 1. According to the results, the studied flavonoids exhibited moderate binding properties towards HSA with essential differences in the binding constants. On the basis of the obtained K values, the binding order could be presented as follows: flavone (21.39×10^3 M^{-1}) > quercitrin (6.64×10^3 M^{-1}) > rutin (4.13×10^3 M^{-1}).

These results could be considered from the viewpoint of the structural peculiarities of the studied flavonoids (Fig. 1). As shown in Table 1, the highest K value belonged to flavone, which represents the basic structure of flavonoids.

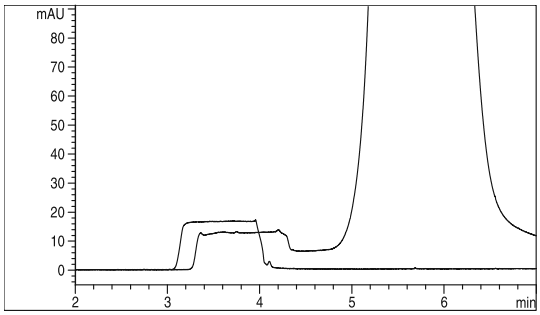

Fig. 3 Electropherograms of 300 μM flavone (–) and 300 μM flavone (+) 40 μM HSA (—). Experimental conditions as in Fig. 2, hydrodynamic injection at 50 mbar for 40 s

In comparison, quercitrin and rutin contain the monoglycoside rhamnose and the diglycoside rutinose (rhamnose + glucose) at the C(3) position, respectively. This fact was reflected by a binding constant about three times lower than flavone in the case of quercitrin and approximately five times lower for rutin. The differences in affinity could be ascribed to the increased molecular size and, as a consequence, less flexible, bulky structures of quercitrin and rutin. This could result in a less favourable placement within the binding site, so that the binding capacity of either quercitrin or rutin is lower than that of flavone. Besides this, the additional polar groups of sugar moieties render these molecules less hydrophobic which could thus effect their orientation in relation to the hydrophobic environment of the HSA. Consequently, it was concluded that the presence of sugar moieties, as well as the number of saccharides, had a marked effect on the flavonoid binding properties to HSA. On the whole, glycosylation of flavonoids decreased their binding capacity to human serum albumin.

The available data obtained by using the conventional method of fluorescence spectroscopy were consistent with the results of the present study. For example, the reported binding constants for rutin obtained by the fluorescence spectroscopy technique were 4.1×10^3 M^{-1} [30], 38×10^3 M^{-1} [31] and variation of $(41.5–105)\times10^3$ M^{-1} depending on the calculation method used [28]. Furthermore, a binding constant for the quercitrin–BSA complex was reported as 6.47×10^3 M^{-1} [32]. The results showed better agreement when the same technique and similar experimental conditions were used. For example, Lu et al., using the CE-FA method, reported a binding constant for the rutin–HSA complex of 1.57×10^3 M^{-1} [33]. Diniz et al. presented binding constants for rutin (6.9×10^3 M^{-1}) and flavone (33×10^3 M^{-1}), obtained by capillary electrophoresis, which were very close to the values presented in the current study [34]. These variations in results arise from differences in experimental conditions and selected methods, as well as from the data processing that follows.

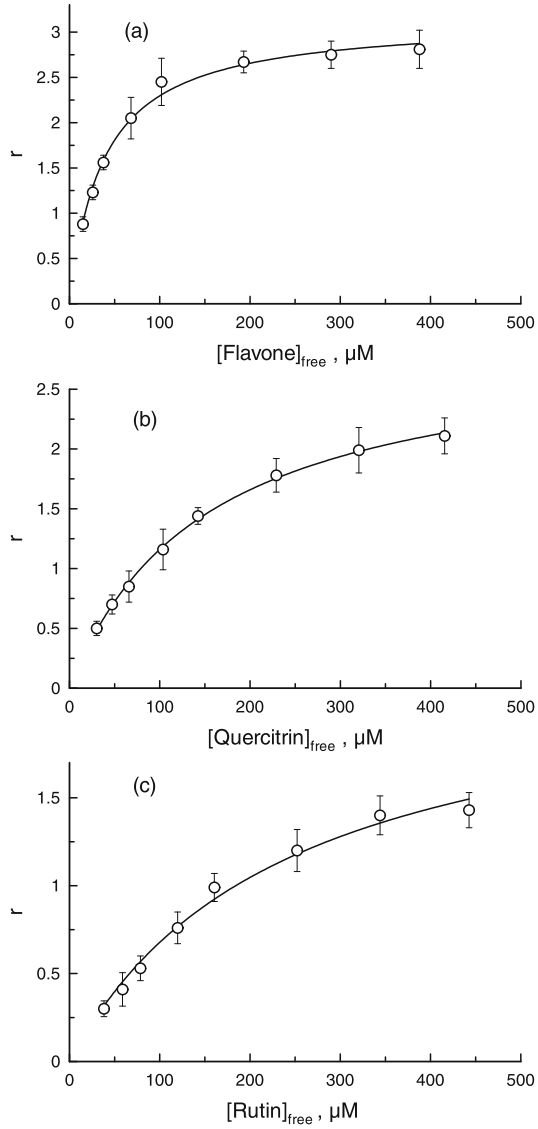

Fig. 4 Binding curves of the studied flavonoids: **a** flavone, **b** quercitrin and **c** rutin

Table 1 Binding parameters obtained by the CE-FA method and lipophilicity (log*P*) values for the studied flavonoids

Compound	K±SD ($\times10^3M^{-1}$)	n±SD[a]	Log*P*
Flavone	21.39±0.55	2.26±0.02	3.737
Quercitrin	6.64±0.10	2.91±0.02	0.644
Rutin	4.13±0.16	2.32±0.04	−1.063

[a] n is the number of primary binding sites

The fluorescence technique used in the example of rutin was examined in an attempt to eliminate some of the factors that may have been responsible for the differences in results. The binding of rutin to HSA was estimated by quenching the intrinsic fluorescence of the protein under similar experimental conditions as used with the CE-FA method. The calculated binding constant of rutin to HSA was $(6.4 \pm 0.8) \times 10^3$ M^{-1}, which was in the same range as the results obtained with CE-FA. This result confirmed that the CE-FA method is adequate for the characterization of ligand–protein binding.

Correlation between the partition coefficient and the binding constant

The partition coefficient (P) describes the propensity of uncharged compounds to differentially dissolve in an immiscible biphasic system (usually organic solvent/water). Referred to as a measure of lipophilicity, the log$P$ value is often used as a key criterion for the prediction and understanding of drug behaviours in the body in early pharmacological studies. Highly lipophilic compounds tend to easily penetrate through certain biological barriers, binding extensively to the protein targets; on the other hand, bioactivity could be compromised due to the low aqueous solubility and the compound may be more likely to accumulate or even affect toxicity [35].

The log$P$ values of flavone, quercitrin and rutin were calculated by using the miLogP software and are presented in Table 1. As can be seen from the data, the lipophilicity (log$P$) of the tested flavonoids was intermediate and decreased after glycosylation. Likewise, the lipophilicity of the flavonoids was correlated with their binding constants, as is shown in Fig. 5. The more hydrophobic flavone showed a high binding constant and could be present in the plasma mainly bound to the protein, improving its solubility and prolonging its half-life. In addition, a marked decrease in the lipophilic characteristics as well as in the binding properties was demonstrated by two glycosides – quercitrin (log$P$=0.644) and rutin (log$P$=−1.063). The results obtained evidently showed that hydrophobic interactions have a significant effect on the binding of flavonoids to HSA.

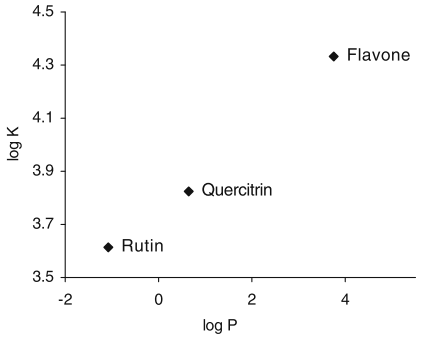

Fig. 5 Relationship between the binding constants (log$K$) of flavonoids and their partition coefficients (log$P$)

Determination of binding site for flavonoids on the HSA

The determination of specific binding sites is usually based on competitive binding, where the studied ligand and the site marker compete for the same binding site on a protein molecule. Compounds with a high binding affinity and selectivity towards the definite binding regions on a protein are used as the site markers. For example, warfarin binds to site I on HSA (K= 1.2 to 7.1×10^5 M^{-1}) and ibuprofen binds to site II (K= 7.1 to 35.6×10^5 M^{-1}) [6]. Thus, warfarin and ibuprofen were used in competitive binding experiments with the studied flavonoids. The molar ratio of ligands to HSA was equal to 1 in order to ensure that binding mainly occurred to the high-affinity sites. The effect of displacement was evaluated by comparing the percentage of flavonoid binding to HSA before and after the addition of site marker.

As demonstrated in Fig. 6, the addition of the site I specific compound (warfarin 100 μM) to the flavonoid (FL)–HSA complex (100 μM FL and 100 μM HSA) resulted in a remarkable reduction in the binding percentage for all of the flavonoids. The decrease was significantly greater (from 41.8 to 23.4%) for rutin in contrast to quercitrin, which demonstrated a reduction in the binding percentage from 72.1 to 58.8%, and flavones, from 90.4 to 80.7%. This implied that there was competition between the flavonoids and warfarin when both existed in the human serum albumin solution at the same time. Then, ibuprofen was tested as the site II marker. In the presence of ibuprofen, no marked changes in the binding properties were observed,

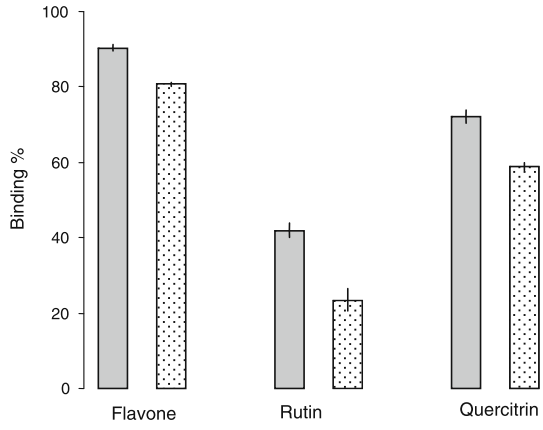

Fig. 6 Representation of competitive binding between the flavonoids and warfarin: ▭ 100 μM flavonoid+100 μM HSA, ▭ 100 μM flavonoid+100 μM HSA+100 μM warfarin

which indicated that ibuprofen did not share the same binding site with flavonoids and independently bound to site II under the studied conditions. Based on these results, it was suggested that the binding site of flavonoids on HSA is at site I. Moreover, it was noted that glycosylation of the flavonoids had no influence on the binding position.

Binding competition between two ligands typically reduces their affinities towards the protein due to partial displacement and/or alteration of protein structure. Under real physiological conditions, the binding of flavonoid to the protein could be affected by the presence of a variety of metabolites or drugs having a similar or higher affinity towards the same binding site on HSA. For example, widely used drugs such as a phenylbutazone, salicylamide, *p*-nitrophenol and quinidine had high *K* values in the range of 10^4–10^5 M^{-1} to the HSA binding site I [6]. Thus, competitive binding could be expected and it could alter the binding of flavonoids to proteins, so the real amount of the active fraction of flavonoids in the plasma may be different than assumed. The ligand–ligand competition for the same binding site is more critical in the case of rutin, which showed the lowest binding affinity to HSA and had the greatest extent of displacement among the studied flavonoids.

Determination of the acting forces between flavonoids and HSA

Hydrogen bonding, van der Waals forces, electrostatic and hydrophobic interactions are the main acting forces between small molecules and proteins. In order to evaluate the interaction forces between the studied flavonoids and HSA, the thermodynamic parameters dependent on temperature were calculated according to the following equations:

$$\ln\frac{K_2}{K_1} = \left(\frac{1}{T_1} - \frac{1}{T_2}\right)\frac{\Delta H}{R}$$

$$\Delta G = -RT\ln K = \Delta H - T\,\Delta S$$

where K_1 and K_2 are the binding constants at the corresponding temperatures T_1 and T_2, R is the gas constant, and ΔH, ΔG and ΔS are the enthalpy, free energy and entropy changes on binding, respectively. If the temperature hardly changes, the enthalpy change is considered as constant.

Based on the above equations, the thermodynamic parameters were calculated and are summarized in Table 2. According to the results, the flavonoid–HSA complex formation was an exothermic reaction ($\Delta H<0$). The negative value of the free energy (ΔG) indicated the spontaneity of the interaction processes, which increased when the temperature increased. Leckband [36], Ross and Subramanian [37] characterized and systematized the sign and magnitude of the thermodynamic parameters related to various protein associations. The positive entropy change ΔS is frequently used as evidence of hydrophobic interactions. Moreover, the dependence of binding constants on the lipophilic characteristics of the studied compounds has been previously demonstrated. Furthermore, rhamnose contains a non-polar methyl group, as can be seen from its structure (Fig. 1), and may contribute to hydrophobic interactions between flavonoid glycosides and HSA. In addition, specific electrostatic interactions are characterized by positive ΔS and negative ΔH values. Molecular modelling and computational mapping of the binding site I of HSA has provided evidence that there are several polar amino acid residues able to stabilize negatively charged molecules [38]. Thus, electrostatic interactions could be expected for rutin and quercitrin due to the presence of partially dissociated hydroxyl groups under the studied conditions. Consequently, it could be assumed that, besides hydrophobic forces, electrostatic interactions play a considerable role in the formation and maintenance of the stability of the complex between HSA and the studied flavonoids. Moreover, the structural similarities of the flavonoids and warfarin suggested that they bound to protein in essentially the same manner. X-ray crystallographic analysis demonstrated that warfarin integrates its aromatic moieties in two distinct sub-pockets forming hydrophobic and aliphatic contacts with the surrounding side chains [39].

Conclusions

The capillary electrophoresis frontal analysis was demonstrated as a persuasive technique for characterization of the binding properties of structurally different flavonoids to HSA. The presented approach allowed the determination of flavonoid–albumin binding constants whilst simultaneously providing

Table 2 Binding constants measured at various temperatures and thermodynamic parameters for flavonoid–HSA complexes

Compound	T (K)	K ($\times10^3 M^{-1}$)	ΔG (kJmol^{-1})	ΔH (kJmol^{-1})	ΔS (Jmol^{-1}K^{-1})
Flavone	298	26.88±1.26	−25.3	−15.3	33.6
	309.5	21.39±0.55	−25.7		
Quercitrin	298	7.96±0.18	−22.3	−12.1	34.1
	309.5	6.64±0.10	−22.6		
Rutin	298	5.02±0.14	−21.1	−13.0	27.2
	309.5	4.13±0.16	−21.4		

information on the binding sites and on the main acting forces for the studied complexes. The binding constants obtained by CE-FA were in agreement with the results of the fluorescence assay, despite the different measurement principles. The obtained results could facilitate the understanding of flavonoid transport in plasma and the prediction of their physiological behaviours by focusing on their structural features. Proven as a simple and rapid technique which uses small sample volumes and which has the potential for automatization, CE-FA was confirmed to be a valuable support or even an alternative to the traditional methods. It could be successfully implemented in analyses and pharmacokinetic studies of new drugs or naturally occurring bioactive compounds.

Acknowledgements The support of the Estonian Science Foundation (Grant No.6166) and the European Social Fund's Doctoral Studies and Internationalisation Programme DoRa activity 8 "Supporting the Participation of Young Researchers in the International Exchange of Knowledge" is acknowledged.

References

1. Barre J, Chamouard JM, Houin G, Tillement JP (1985) Equilibrium dialysis, ultrafiltration, and ultracentrifugation compared for determining the plasma-protein-binding characteristics of valproic acid. Clin Chem 31:60–64
2. Lakowicz JR (2006) Principles of fluorescence spectroscopy, 3rd edn. Springer-Verlag, New York Inc
3. Dalvit C, Hadden D, Sarver RW, Ho AM, Stockman BJ (2003) Multi-selective one dimensional proton NMR experiments for rapid screening and binding affinity measurements. Comb Chem High Throughput Screen 6(5):445–453
4. Myszka DG, Rich RL (2000) Implementing surface plasmon resonance biosensors in drug discovery. Pharm Sci Technol Today 3:310–317
5. Østergaard J, Heegaard N (2003) Capillary electrophoresis frontal analysis: principles and applications for the study of drug-plasma protein binding. Electrophoresis 24:2903–2913
6. Østergaard J, Schou C, Larsen C, Heegaard N (2002) Evaluation of capillary electrophoresis-frontal analysis for the study of low molecular weight drug-human serum albumin interactions. Electrophoresis 23:2842–2853
7. Martínez-Gómez MA, Sagrado S, Villanueva-Camañas RM, Medina-Hernández MJ (2006) Characterization of basic drug-human serum albumin interactions by capillary electrophoresis. Electrophoresis 27:3410–3419
8. Jia Z, Ramstand T, Zhong M (2002) Determination of protein-drug binding constants by pressure-assisted capillary electrophoresis (PACE)/frontal analysis (FA). J Pharm Biomed Anal 30:405–413
9. Peters T (1995) All about albumin: biochemistry, genetics, and medical applications. Academic, San Diego
10. Varshney A, Sen P, Ahmad E, Rehan M, Subbarao N, Khan RH (2010) Ligand binding strategies of human serum albumin: how can the cargo be utilized? Chirality 22:77–87
11. Sudlow G, Birkett DJ, Wade DN (1975) The characterization of two specific drug binding sites on human serum albumin. Mol Pharmacol 11:824–832
12. Sudlow G, Birkett DJ, Wade DN (1976) Further characterization of specific drug binding sites on human serum albumin. Mol Pharmacol 12:1052–1061
13. Ghuman J, Zunszain PA, Petitpas I, Bhattacharya AA, Otagiri M, Curry S (2005) Structural basis of the drug-binding specifity of human serum albumin. J Mol Biol 353:38–52
14. Aleksenko S, Hartinger C, Semenova O, Meelich K, Timerbaev A, Keppler B (2007) Characterization of interactions between human serum albumin and tumor-inhibiting amino alcohol platinum (II) complexes using capillary electrophoresis. J Chromatogr A 1155:218–221
15. Bi S, Song D, Tian Y, Zhhou X, Liu Z, Zhang H (2005) Molecular spectroscopic study on the interaction of tetracyclines with serum albumins. Spectrochim Acta A Mol Biomol Spectrosc 61:629–636
16. Sun H, He P (2009) Characterization of interaction between doxycycline and human serum albumin by capillary electrophoresis-frontal analysis. Electrophoresis 30:1991–1997
17. Liu Y, Xie MX, Jiang M, Wang YD (2005) Spectroscopic investigation of the interaction between human serum albumin and three organic acids. Spectrochim Acta Part A 61:2245–2251
18. Cook NC, Samman S (1996) Flavonoids—chemistry, metabolism, cardioprotective effects, and dietary sources. J Nutr Biochem 7:66–76
19. Shahidi F (1997) Natural antioxidants—chemistry, health effects and applications. AOCS, Champaign
20. Rusznyàk S, Szent-Gyorgyi A (1936) Vitamin P: flavonols as vitamin. Nature 138:27
21. Arnoldi A (2004) Functional foods, cardiovascular disease and diabetes. Woodhead, Cambridge
22. Yao LH, Jiang YM, Shi J, Tomás-Barberán FA, Datta N, Singanusong R, Chen SS (2004) Flavonoids in food and their health benefits. Plant Foods Hum Nutr 59:113–122
23. Eberhardt MV, Lee CY, Liu RH (2000) Antioxidant activity of fresh apples. Nature 405:903–904
24. Helmja K, Vaher M, Püssa T, Kaljurand M (2009) Analysis of the stable free radical scavenging capability of artificial polyphenol mixtures and plant extracts by capillary electrophoresis and liquid chromatography-diod array detection-tandem mass spectrometry. J Chromatogr A 1216:2417–2423
25. Li N, Liu J-H, Zhang J, Yu B-Y (2008) Comparative evaluation of cytotoxicity and antioxidative activity of 20 flavonoids. J Agric Food Chem 56(10):3876–3883
26. Molinspiration Cheminformatics (2005) miLogP version 2.2. Available via http://www.molinspiration.com , Accessed Sept 2009
27. Tanaka Y, Terabe S (2002) Estimation of binding constants by capillary electrophoresis. J Chromatogr B 768:81–92
28. Bi S, Ding L, Tian Y, Song D, Zhou X, Liu X, Zhang H (2004) Investigation of the interaction between flavonoids and human serum albumin. J Mol Struct 703:37–45
29. Lemanska K, Szymusiak H, Tyrakowska B, Zieliski R, Soffers A, Rietjens I (2001) The influence of pH on antioxidant properties and the mechanism of antioxidant action of hydroxyflavones. Free Radic Biol Med 31:869–881
30. Luan NN, Wu JX, Song YM, Wu Q (2008) Thermodynamic studies on the binding of rutin and serum albumin. Guang Pu Xue Yu Guang Pu Fen Xi 28(4):856–859
31. Pastukhov AV, Levchenko LA, Sadkov AP (2007) Spectroscopic study on binding of rutin to human serum albumin. J Molec Struct 842:60–66
32. Cao H, Wu D, Wang H, Xu M (2008) Influence of the B-ring hydroxylation on interactions of flavonols with bovine serum albumin. J Agric Food Chem 56:2350–2356
33. Q-h Lu, Ba C, Chen D (2008) Investigating noncovalent interactions of rutin–serum albumin by capillary electrophoresis–frontal analysis. J Pharm Biomed Anal 47:888–891
34. Diniz A, Escuder-Gilabert L, Lopes NP, Villanueva-Camanas RM, Sagrado S, Medina-Hernandez MJ (2008) Characterization of interactions between polyphenolic compounds and human serum proteins by capillary electrophoresis. Anal Bioanal Chem 391:625–632

35. Sangster J (1997) Octanol-water partition coefficients: fundamentals and physical chemistry, vol 2. Wiley, Chichester
36. Leckband D (2000) Measuring the forces that control protein interactions. Annu Rev Biophys Biomol Struct 29:1–26
37. Ross DP, Subramanian S (1981) The thermodynamics of protein association reactions: forces contributing to stability. Biochemistry 20:3096–3102
38. Zsila F, Bikádi Z, Simonyi M (2003) Probing the binding of the flavonoid, quercetin to himan serum albumin by circular dichroism, electronic absorption spectroscopy and molecular modelling methods. Biochem Pharmacol 65:447–456
39. Petitpas I, Bhattacharya AA, Twine S, East M, Curry S (2001) Crystal structure analysis of warfarin binding to human serum albumin. J Biol Chem 276:22804–22809

CURRICULUM VITAE

Personal data

Name	Tatjana Knjazeva
Date of birth	25.07.1981
Citizenship	Estonian
Family status	Married

Contact information

Address	Tallinn University of Technology (TUT), Department of Chemistry, Akadeemia tee 15-325, 12618 Tallinn, Estonia
Phone	+3726204322
E-mail	tatjana.knjazeva@ttu.ee

Education

TUT, 2006, chemical and materials science, M.Sc.
TUT, 2003, bio- and food technology, B.Sc.
Tallinn Pae Gymnasium, 1999, secondary education

Professional employment

September 2010 -	TUT, Department of Chemistry, researcher
2007 – 2010	TUT, Chair of Analytical Chemistry, scientific research in the implementation of projects under the Estonian Ministry of Defense
2007 – 2009	Teaching in the field of inorganic chemistry

Special courses

2009 -	Graduate school "Functional materials and processes"
2006 – 2008	Graduate school "New production technologies and processes"

Defended thesis

Master's thesis "Analysis of phosphonic compounds by capillary electrophoresis with contactless conductivity detection", 2006.
Bachelor's thesis "Supercritical extraction of rosehips", 2003.

Theses supervised Eeva-Gerda Kobrin, B.Sc. (TUT, 2009)

Main areas of scientific work/Current research topics

Capillary electrophoresis, analytical system miniaturization, environmental and biological samples analysis

List of original publications

1) T. Komissarova, M. Kulp. Analysis of CWA degradation products by portable capillary electrophoretic analyser. *Proc. 31th International*

Symposium on High Performance Liquid Phase Separations and Related Techniques, Gent, Belgium, 2007
2) M. Kulp, T. Komissarova, M. Kaljurand. Electrophoretic analysis of biological anions and proteins in polyether ether ketone capillaries with capacitively coupled contactless conductivity detection. *Proc. 12th International meeting on recent developments in pharmaceutical analysis, Island of Elba, Italy, 2007.*
3) T. Komissarova, M. Kulp, M. Kaljurand. Application of Polyether Ether Ketone Capillary for Electrophortic Separation of Complicated Biological Samples. *Proc. 22nd International Symposium on MicroScale Bioseparatioms & Methods for System Biology, Berlin, German, 2008.*
4) T. Knjazeva, M. Kulp, M. Kaljurand. CE separation of various analytes of biological origin using polyether ether ketone capillaries and contactless conductivity detection. *Electrophoresis* 2009, 30, 424-430.
5) J. Mazina, J. Gorbatsova, T. Knjazeva, M. Kaljurand. Simultaneous determination of water-soluble vitamins in food samples by CE coupled with MALDI. *Proc. Natural Products Conference, Antigua, 2009.*
6) K. Helmja, M. Borissova, T. Knjazeva, M. Jaanus, U. Muinasmaa, M. Kaljurand, M. Vaher. Fraction collection in capillary electrophoresis for various stand-alone mass spectrometers. *J. Chromatogr.* A 2009, 1216, 3666-3673.
7) T. Knjazeva, E.-G. Kobrin, M. Kaljurand. Capillary electrophoresis frontal analysis for the study of flavonoid noncovalent interactions with human serum albumin. *Proc. 5th Conference on Separation and Related Techniques by Nordic Separation Science Society, Tallinn, Estonia, 2009.*
8) Knjazeva Tatjana, Kaljurand Mihkel, Interactions between bioactive flavonoids and human serum albumin: determination of binding constants, binding sites and thermodynamic parameters by capillary electrophoresis. *Proc. 6th International Conference "Instrumental Methods of Analysis, Modern Trends and Applications", Athens, Greece, 2009.*
9) T. Knjazeva, N. Makarõtševa, E.-G. Kobrin, K. Helmja, A. Seiman, M. Vaher, M. Kaljurand. Portatiivne kapillaarelektroforeesi aparaat keskkonnareostuse analüüsiks. *Proc. 31th Estonian Chemistry Days, Tallinn, Estonia, 2010.*
10) T. Knjazeva, M. Kaljurand. Capillary electrophoresis frontal analysis for the study of flavonoid interactions with human serum albumin. *Anal. Bioanal. Chem.* 2010, 397, 2211-2219.

ELULOOKIRJELDUS

Isikuandmed

Ees- ja perekonnanimi	Tatjana Knjazeva
Sünniaeg ja -koht	25.07.1981, Valgevene
Kodakondsus	Eesti
Perekonnaseis	Abielus

Kontaktandmed

Aadress	Tallinna Tehnikaülikool (TTÜ), Keemiainstituut, Akadeemia tee 15-325, 12618 Tallinn, Eesti
Telefon	+3726204322
E-posti aadress	tatjana.knjazeva@ttu.ee

Hariduskäik

TTÜ, 2006, keemia- ja materjaliteadus, loodusteaduste magister
TTÜ, 2003, bio- ja toiduainetehnoloogia, loodusteaduste bakalaureus
Tallinna Pae Gümnaasium, 1999, keskharidus

Teenistuskäik

September 2010 -	TTÜ, Keemiainstituut, teadur
2007 - 2010	TTÜ, Analüütilise keemia õppetool, teadus-uurimistöö EV Kaitseministeeriumi projektide täitmise raames
2007 - 2009	Õppetöö läbiviimine anorgaanilise keemia alal

Täiendusõpe

2009 -	Doktorikool "Funktsionaalsed materjalid ja tehnoloogiad"
2006 – 2008	Uute tootmistehnoloogiate ja -protsesside doktorikool

Kaitstud lõputööd

Magistritöö "Fosfoonühendite kapillaarelektroforeetiline analüüs kasutades kontaktita juhtivusdetektorit", 2006.
Bakalaureusetöö "Kibuvitsamarjade ülekriitiline ekstraktsioon", 2003.

Teadustöö põhisuunad

Kapillaarelektroforees, analüütiliste meetodite miniaturiseerimine, keskkonna- ja bioloogiliste proovide uurimine

DISSERTATIONS DEFENDED AT TALLINN UNIVERSITY OF TECHNOLOGY ON *NATURAL AND EXACT SCIENCES*

1. **Olav Kongas**. Nonlinear dynamics in modeling cardiac arrhytmias. 1998.

2. **Kalju Vanatalu**. Optimization of processes of microbial biosynthesis of isotopically labeled biomolecules and their complexes. 1999.

3. **Ahto Buldas**. An algebraic approach to the structure of graphs. 1999.

4. **Monika Drews**. A metabolic study of insect cells in batch and continuous culture: application of chemostat and turbidostat to the production of recombinant proteins.1999.

5. **Eola Valdre**. Endothelial-specific regulation of vessel formation: role of receptor tyrosine kinases. 2000.

6. **Kalju Lott**. Doping and defect thermodynamic equilibrium in ZnS. 2000.

7. **Reet Koljak**. Novel fatty acid dioxygenases from the corals *Plexaura homomalla* and *Gersemia fruticosa.* 2001.

8. **Anne Paju**. Asymmetric oxidation of prochiral and racemic ketones by using sharpless catalyst. 2001.

9. **Marko Vendelin**. Cardiac mechanoenergetics *in silico*. 2001.

10. **Pearu Peterson**. Multi-soliton interactions and the inverse problem of wave crest. 2001.

11. **Anne Menert**. Microcalorimetry of anaerobic digestion. 2001.

12. **Toomas Tiivel**. The role of the mitochondrial outer membrane in *in vivo* regulation of respiration in normal heart and skeletal muscle cell. 2002.

13. **Olle Hints**. Ordovician scolecodonts of Estonia and neighbouring areas: taxonomy, distribution, palaeoecology, and application. 2002.

14. **Jaak Nõlvak**. Chitinozoan biostratigrapy in the Ordovician of Baltoscandia. 2002.

15. **Liivi Kluge**. On algebraic structure of pre-operad. 2002.

16. **Jaanus Lass**. Biosignal interpretation: Study of cardiac arrhytmias and electromagnetic field effects on human nervous system. 2002.

17. **Janek Peterson**. Synthesis, structural characterization and modification of PAMAM dendrimers. 2002.

18. **Merike Vaher**. Room temperature ionic liquids as background electrolyte additives in capillary electrophoresis. 2002.

19. **Valdek Mikli**. Electron microscopy and image analysis study of powdered hardmetal materials and optoelectronic thin films. 2003.

20. **Mart Viljus**. The microstructure and properties of fine-grained cermets. 2003.

21. **Signe Kask**. Identification and characterization of dairy-related *Lactobacillus.* 2003.

22. **Tiiu-Mai Laht**. Influence of microstructure of the curd on enzymatic and microbiological processes in Swiss-type cheese. 2003.

23. **Anne Kuusksalu**. 2–5A synthetase in the marine sponge *Geodia cydonium*. 2003.

24. **Sergei Bereznev**. Solar cells based on polycristalline copper-indium chalcogenides and conductive polymers. 2003.

25. **Kadri Kriis**. Asymmetric synthesis of C2-symmetric bimorpholines and their application as chiral ligands in the transfer hydrogenation of aromatic ketones. 2004.

26. **Jekaterina Reut**. Polypyrrole coatings on conducting and insulating substracts. 2004.

27. **Sven Nõmm**. Realization and identification of discrete-time nonlinear systems. 2004.

28. **Olga Kijatkina**. Deposition of copper indium disulphide films by chemical spray pyrolysis. 2004.

29. **Gert Tamberg**. On sampling operators defined by Rogosinski, Hann and Blackman windows. 2004.

30. **Monika Übner**. Interaction of humic substances with metal cations. 2004.

31. **Kaarel Adamberg**. Growth characteristics of non-starter lactic acid bacteria from cheese. 2004.

32. **Imre Vallikivi**. Lipase-catalysed reactions of prostaglandins. 2004.

33. **Merike Peld**. Substituted apatites as sorbents for heavy metals. 2005.

34. **Vitali Syritski**. Study of synthesis and redox switching of polypyrrole and poly(3,4-ethylenedioxythiophene) by using *in-situ* techniques. 2004.

35. **Lee Põllumaa**. Evaluation of ecotoxicological effects related to oil shale industry. 2004.

36. **Riina Aav**. Synthesis of 9,11-secosterols intermediates. 2005.

37. **Andres Braunbrück**. Wave interaction in weakly inhomogeneous materials. 2005.

38. **Robert Kitt**. Generalised scale-invariance in financial time series. 2005.

39. **Juss Pavelson**. Mesoscale physical processes and the related impact on the summer nutrient fields and phytoplankton blooms in the western Gulf of Finland. 2005.

40. **Olari Ilison**. Solitons and solitary waves in media with higher order dispersive and nonlinear effects. 2005.

41. **Maksim Säkki**. Intermittency and long-range structurization of heart rate. 2005.

42. **Enli Kiipli**. Modelling seawater chemistry of the East Baltic Basin in the late Ordovician–Early Silurian. 2005.

43. **Igor Golovtsov**. Modification of conductive properties and processability of polyparaphenylene, polypyrrole and polyaniline. 2005.

44. **Katrin Laos**. Interaction between furcellaran and the globular proteins (bovine serum albumin β-lactoglobulin). 2005.

45. **Arvo Mere**. Structural and electrical properties of spray deposited copper indium disulphide films for solar cells. 2006.

46. **Sille Ehala**. Development and application of various on- and off-line analytical methods for the analysis of bioactive compounds. 2006.

47. **Maria Kulp**. Capillary electrophoretic monitoring of biochemical reaction kinetics. 2006.

48. **Anu Aaspõllu.** Proteinases from *Vipera lebetina* snake venom affecting hemostasis. 2006.

49. **Lyudmila Chekulayeva**. Photosensitized inactivation of tumor cells by porphyrins and chlorins. 2006.

50. **Merle Uudsemaa**. Quantum-chemical modeling of solvated first row transition metal ions. 2006.

51. **Tagli Pitsi**. Nutrition situation of pre-school children in Estonia from 1995 to 2004. 2006.

52. **Angela Ivask**. Luminescent recombinant sensor bacteria for the analysis of bioavailable heavy metals. 2006.

53. **Tiina Lõugas**. Study on physico-chemical properties and some bioactive compounds of sea buckthorn (*Hippophae rhamnoides* L.). 2006.

54. **Kaja Kasemets**. Effect of changing environmental conditions on the fermentative growth of Saccharomyces cerevisae S288C: auxo-accelerostat study. 2006.

55. **Ildar Nisamedtinov**. Application of 13C and fluorescence labeling in metabolic studies of Saccharomyces spp. 2006.

56. **Alar Leibak**. On additive generalisation of Voronoï's theory of perfect forms over algebraic number fields. 2006.

57. **Andri Jagomägi**. Photoluminescence of chalcopyrite tellurides. 2006.

58. **Tõnu Martma**. Application of carbon isotopes to the study of the Ordovician and Silurian of the Baltic. 2006.

59. **Marit Kauk**. Chemical composition of CuInSe 2 monograin powders for solar cell application. 2006.

60. **Julia Kois**. Electrochemical deposition of CuInSe2 thin films for photovoltaic applications. 2006.

61. **Ilona Oja Açik**. Sol-gel deposition of titanium dioxide films. 2007.

62. **Tiia Anmann**. Integrated and organized cellular bioenergetic systems in heart and brain. 2007.

63. **Katrin Trummal**. Purification, characterization and specificity studies of metalloproteinases from *Vipera lebetina* snake venom. 2007.

64. **Gennadi Lessin**. Biochemical definition of coastal zone using numerical modeling and measurement data. 2007.

65. **Enno Pais**. Inverse problems to determine non-homogeneous degenerate memory kernels in heat flow. 2007.

66. **Maria Borissova**. Capillary electrophoresis on alkylimidazolium salts. 2007.

67. **Karin Valmsen**. Prostaglandin synthesis in the coral *Plexaura homomalla*: control of prostaglandin stereochemistry at carbon 15 by cyclooxygenases. 2007.

68. **Kristjan Piirimäe**. Long-term changes of nutrient fluxes in the drainage basin of the gulf of Finland – application of the PolFlow model. 2007.

69. **Tatjana Dedova**. Chemical spray pyrolysis deposition of zinc sulfide thin films and zinc oxide nanostructured layers. 2007.

70. **Katrin Tomson**. Production of labelled recombinant proteins in fed-batch systems in *Escherichia coli*. 2007.

71. **Cecilia Sarmiento**. Suppressors of RNA silencing in plants. 2008.

72. **Vilja Mardla**. Inhibition of platelet aggregation with combination of antiplatelet agents. 2008.

73. **Maie Bachmann**. Effect of Modulated microwave radiation on human resting electroencephalographic signal. 2008.

74. **Dan Hüvonen**. Terahertz spectroscopy of low-dimensional spin systems. 2008.

75. **Ly Villo**. Stereoselective chemoenzymatic synthesis of deoxy sugar esters involving *Candida antarctica* lipase B. 2008.

76. **Johan Anton**. Technology of integrated photoelasticity for residual stress measurement in glass articles of axisymmetric shape. 2008.

77. **Olga Volobujeva**. SEM study of selenization of different thin metallic films. 2008.

78. **Artur Jõgi**. Synthesis of 4'-substituted 2,3'-dideoxynucleoside analogues. 2008.

79. **Mario Kadastik**. Doubly charged Higgs boson decays and implications on neutrino physics. 2008.

80. **Fernando Pérez-Caballero**. Carbon aerogels from 5-methylresorcinol-formaldehyde gels. 2008.

81. **Sirje Vaask**. The comparability, reproducibility and validity of Estonian food consumption surveys. 2008.

82. **Anna Menaker**. Electrosynthesized conducting polymers, polypyrrole and poly(3,4-ethylenedioxythiophene), for molecular imprinting. 2009.

83. **Lauri Ilison**. Solitons and solitary waves in hierarchical Korteweg-de Vries type systems. 2009.

84. **Kaia Ernits**. Study of In2S3 and ZnS thin films deposited by ultrasonic spray pyrolysis and chemical deposition. 2009.

85. **Veljo Sinivee**. Portable spectrometer for ionizing radiation "Gammamapper". 2009.

86. **Jüri Virkepu**. On Lagrange formalism for Lie theory and operadic harmonic oscillator in low dimensions. 2009.

87. **Marko Piirsoo**. Deciphering molecular basis of Schwann cell development. 2009.

88. **Kati Helmja**. Determination of phenolic compounds and their antioxidative capability in plant extracts. 2010.

89. **Merike Sõmera**. Sobemoviruses: genomic organization, potential for recombination and necessity of P1 in systemic infection. 2010.

90. **Kristjan Laes**. Preparation and impedance spectroscopy of hybrid structures based on CuIn3Se5 photoabsorber. 2010.

91. **Kristin Lippur**. Asymmetric synthesis of 2,2'-bimorpholine and its 5,5'-substituted derivatives. 2010.

92. **Merike Luman**. Dialysis dose and nutrition assessment by an optical method. 2010.

93. **Mihhail Berezovski**. Numerical simulation of wave propagation in heterogeneous and microstructured materials. 2010.

94. **Tamara Aid-Pavlidis**. Structure and regulation of BDNF gene. 2010.

95. **Olga Bragina**. The role of Sonic Hedgehog pathway in neuro- and tumorigenesis. 2010.

96. **Merle Randrüüt**. Wave propagation in microstructured solids: solitary and periodic waves. 2010.

97. **Marju Laars**. Asymmetric organocatalytic Michael and aldol reactions mediated by cyclic amines. 2010.

98. **Maarja Grossberg**. Optical properties of multinary semiconductor compounds for photovoltaic applications. 2010.

99. **Alla Maloverjan**. Vertebrate homologues of Drosophila fused kinase and their role in Sonic Hedgehog signalling pathway. 2010.

100. **Priit Pruunsild**. Neuronal Activity-Dependent Transcription Factors and Regulation of Human *BDNF* Gene. 2010.