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MULTIDIMENSIONAL SEPARATION METHODS IN ANALYSIS OF COMPLEX BIOLOGICAL MIXTURES

Ph.D. Dissertation

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Pledge

I hereby confirm that I have entirely worked out this thesis by myself, using techniques and literature described herein.

December 20th, 2013 ....

Brno, Czech Republic Mgr. Filip Duša

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ABSTRACT

This work deals with two-dimensional separations of peptides and proteins from biological matrices. Theoretical introduction describes extraction of proteins from biological matrices, modes of isoelectric focusing (IEF) and liquid chromatography (LC) used for fractionation in two-dimensional separation schemes, and current papers dealing with combination of IEF and LC. In the results chapter there are four papers, which passed reviews and have been published in international scientific journals. In the first paper, divergent-flow IEF was evaluated for separation of peptides in the mode of isoelectric autofocusing. Capillary LC was used as the second dimension to resolve fractions obtained by autofocusing. Furthermore, novel micropreparative IEF device was described and used for two-dimensional separation of proteins and peptides from whey in following article. Simple splitless nano column LC instrument was presented in the third paper and tested for gradient elution in nano volumes. Finally, power supply adapted for strip format of IEF was presented in the last research paper. In this work I have shown that combination of IEF and LC in two-dimensional schemes is still topical and further improvements in both separation techniques are beneficial for separation of proteins and peptides contained in biological mixtures.

ABSTRACT (In Czech)

Tato práce se zabývá dvoudimenzionálními separacemi peptidů a proteinů obsažených v biologických matricích. Teoretický úvod popisuje extrakci proteinů z biologických matricí, módy izoelektrické fokusace (IEF) a kapalinové chromatografie (LC) používané pro frakcionaci ve dvoudimenzionálních separačních schématech a aktuální články zabývající se kombinací IEF a LC. V části výsledků jsou uvedeny čtyři vědecké články, které prošly připomínkovým řízením a byly publikovány v mezinárodních vědeckých časopisech. V prvním článku bylo testováno zařízení pro IEF v rozbíhavém toku na separaci peptidů pomocí módu autofokusace. Kapilárním formát LC v druhé dimenzi byl následně použit pro separaci frakcí získaných pomocí autofokusace. V následujícím článku bylo popsáno zařízení pro mikropreparativní analýzu pomocí IEF a následně bylo toto zařízení využito pro dvoudimenzionální separaci proteinů a peptidů ze syrovátky. Jednoduchý kapalinový chromatograf využívající tvorbu gradientu mobilní fáze bez dělení průtoku byl popsán ve třetím článku a otestován pro gradientovou eluci v nano objemech. V posledních článku byl popsán zdroj elektrického proudu upravený pro využití při IEF se separačním ložem ve tvaru proužku. V této práci jsem ukázal, že kombinace IEF a LC do dvoudimenzionálních schémat je stále živým tématem a další zlepšování obou metod je přínosné pro separace peptidů a proteinů obsažených v biologických matricích.

LIST OF ABBREVIATIONS

2D	two-dimensional
BM	biological matrices
BSA	bovine serum albumin
CA	carrier ampholytes
CIEF	capillary isoelectric focusing
CEC	capillary electrochromatography
CMP	caseinomacropeptide
CZE	capillary zone electrophoresis
DF-IEF	divergent-flow isoelectric focusing device
ESI	electrospray ionization
ERLIC	electrostatic repulsion hydrophobic interaction chromatography
FFE	free flow electrophoresis
HILIC	hydrophilic interaction chromatography
HV	high voltage
IEF	isoelectric focusing
IPG	immobilized pH gradient
LC	liquid chromatography
m	molecular mass
MF	mobile phase
MS	mass spectrometry
MSWIFT	membrane-separated wells for isoelectric focusing and trapping
P	peak capacity
p*I*	isoelectric point
PIEF	paper-based IEF

RAM	restricted access material
RPLC	reversed phase liquid chromatography
SAX	strong anion-exchange chromatography
SCX	strong cation-exchange chromatography
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
UV	ultraviolet
UV DAD	ultraviolet wavelength diode array detector

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FOREWORD

The nature has been source of constant wonderment ever since the people have started to think how things are working. This amazement and desire for unravelling nature puzzles was melted through the years into the whole brand of human activity - science. People very soon realized that uncovering secrets of the nature brings many advantages. Nowadays, the science is of such importance that almost each country in the world is funding the scientists to benefit from their findings and innovations. With this work, I would like to add another piece to ever increasing human knowledge.

The need of powerful and reliable methods capable of separating minute amounts of analytes rose with continually increasing number of labs investigating biological matrices. Biological samples include for example crude extracts of plant, animal, or fungi tissues and in other cases presence of cultivating medium in sample can be expected when cells or tissues are grown artificially. At this level, it is crucial to remove ballast substances, which do not provide any usable information and which are frequently present in high concentrations. A broad range of methods for removal of unwanted matrix and basic pre-separation/extraction is available (e.g. protein/nucleic acid precipitation, ultrafiltration, centrifugation etc.). These methods are usually specific for diverse kinds of analyte types. After those first steps, which are usually working with higher volumes of samples (commonly milliliters), come more delicate separation methods where separation efficiency is more important than sample throughput. These can be sometimes combined into multi-dimensional systems to get higher separation efficiency and to benefit from more physical/chemical properties in which analytes can differ. In this work, the two-dimensional

(2D) separation platforms consisting of isoelectric focusing in the first dimension off line coupled to high performance liquid chromatography in the second dimension are evaluated. Suggested combination of techniques was able to process crude sample at the input and to give valuable data about composition of the sample at the output.

1 GENERAL INTRODUCTION

1.1 Complex biological matrices

This part includes brief description of various samples used in the field of biological research and major obstacles which prevent their analysis. In addition, an overview of basic setups for decreasing complexity of the sample (fractionation/pre-separation) will be provided.

Biological matrices (BM) differ substantially according to their origin. They often consist of tissues, cells or even organelles. What they have in common is that BM usually contain substances from three groups such as: nucleic acids, metabolites and proteins. Nucleic acids had been intensively investigated in the two last decades of the twentieth century, which resulted into unraveling of complete human genome and complete genomes of many more species. Great number of nucleic acids separation and purification methods were discovered during those years. Metabolites are mostly small molecules with molecular mass (m) up to few hundreds Da which are studied in metabolomics. Metabolomics has become popular quite recently as a tool for recognition of organisms' overall condition, state of specific biochemical pathways, and state of some diseases along with their progress/recess. Finally, the ongoing extensive research of proteins (proteomics) is focused mainly on discovery of new biomarkers for various diseases including great portion of cancer biomarkers. Moreover, proteomics helps scientists to understand many physiological processes in nature and to investigate web of their connections throughout the cell. This thesis is focused on the separation methods of the last group – proteins, which are discussed in detail in the thesis.

1.1.1 Protein extraction and solubilization

Usually, proteins from biological matrices come in native conformation and they are also enclosed inside a cell or in cellular membrane. Therefore, the first step is a membrane disruption and subsequent cell lysis which might be divided into five methods: mechanical disruption, ultrasonication, pressure induced disruption, freeze-thaw cycles and osmotic/detergent lysis[1]. Proteins obtained from those lysates are often present in multi-protein complexes or may be insoluble due to their native form. Moreover native conformation of proteins may hide some amino acid functional groups from protein surface and thus alter results of separation methods based on primary structure of proteins. For that reason protein solubilization techniques are used. They include addition of chaotropic agents, detergents, or reducing agents. In this step, protease inhibitors are also frequently added to prevent any sample losses caused by enzyme cleavage. Chaotropic agents disrupt weak forces which hold secondary structures of proteins and the result is unfolding of proteins. Detergents help to solubilize proteins, but they must be used with regard to the further applied separation methods (e.g. sodium dodecyl sulphate (SDS) prevents separation according to isoelectric point in subsequent analysis). Reducing of disulfide bonds enables complete unfolding of the protein and consequent alkylation of sulfhydryl groups hinders future formation of random disulfide bonds[1]. When the above mentioned steps are carried out, the following step is a removal of contaminants from the obtained mixture.

1.1.2 Removal of contaminants

Apart from proteins, biological matrices contain distinct varieties of substances which can interfere with the separation of the proteins and used detection techniques. Detailed review article dealing with sample preparation for proteomic analysis have been published recently[1].

Contaminants include salts, buffers, detergents, nucleic acids, polysaccharides, lipids and particulates. Salts and substances with small molecules can be easily removed by dialysis. In contrast to laborious and time consuming classical dialysis approach there are several new methods such as spin microdialysis, ultrafiltration, gel filtration, precipitation of proteins with trichloroacetic acid or organic solvents, and solid-phase extraction which can significantly shorten contaminants removal time. Extraction of salt from sample can be also performed by IEF or by using of reversed phase materials (e.g. pipette tips, microcolumns, centrifugal filters). Detergents can be removed using dialysis, gel filtration chromatography, hydrophobic absorption chromatography or protein precipitation. Lipids and polysaccharides are most commonly removed by protein precipitation whereas nucleic acids are often precipitated and centrifuged to a pellet. Centrifugation is also method of choice for possible particulates occurring in sample[1].

1.1.3 Protein fractionation

In further step, which follows after the removal of contaminants from a biological sample, proteins are concentrated and pre-separated (into several fractions). Fractionation of a sample is crucial for reduction of protein dynamic range and their number. Without fractionation, we would not be able to detect plenty of proteins due to limits of separation and detection method. Choice of a pre-separation method is basically determined by a sample composition. These methods include filtration, centrifugation and precipitation techniques along with electrophoresis and liquid chromatography.

Filtration is used in specific applications for separation of peptides based on their molecular weight. A 10 kDa cut-off centrifugation filter was used

for extraction of proteins from bacterial lysates with consequent tryptic digestion of peptides in the filter. After digestion, filter was centrifuged and authors get fraction of tryptic peptides free from trypsin[2]. In other study centrifugation filter with cut-off 30 kDa was used for fractionation of low molecular weight proteins bound to larger proteins[3].

Ultracentrifugation at sucrose/mannitol gradient is often used for fractionation of subcellular compartments including mitochondria, Golgi apparatus, Nuclei, cellular membrane, and other which are separated according to density characteristics of the structure[1,4]. Plasma lipoproteins were effectively fractionated in D_2O/sucrose and KBr gradients. The authors of this study reported that usage of D_2O/sucrose had certain benefits of unnecessary desalting and more complete characterization of apolipoproteins and proteins compared to usage of KBr gradient[5].

Different substances can be used to precipitate proteins from the sample. Namely they are ethanol, isopropanol, acetone, diethyl ether, chloroform/methanol, ammonium sulphate, trichloroacetic acid, and polyethylene glycol[1,6,7]. Immunoprecipitation based on interaction of antibody with specific antigen[8] unlike above mentioned unspecific protein precipitations mentioned above is another possibility how to precipitate protein.

The first three fractionation techniques can produce only a few fractions based on their principle. On the contrary, electrophoretic and chromatographic methods can produce up to tens of fractions and therefore they tend to be more separation than fractionation techniques and as a consequence, they are frequently incorporated into multidimensional separation systems. For this reason and due to a variety of their modes, they are deeply discussed in separate chapters.

1.2 Isoelectric focusing

Isoelectric focusing relies on pH gradient formed in separation bed when high voltage (HV) is applied. External carrier ampholytes or ampholytes already contained in a sample can be used for pH gradient formation. Separation beds in IEF include various shapes and they can be based on different materials. In addition, HV time course should be in particular controlled throughout the analysis.

1.2.1 Carrier ampholytes

One of the crucial things in IEF is a formation of a pH gradient and carrier ampholytes (CA) are usually employed for such task. CA were firstly developed by Vesterberg in 1960s[9]. He succeeded with synthesis of CA from organic amines. Moreover, with further improvement and optimization he was able to produce mixtures of CA capable of creating almost linear pH gradient by the end of 1960s. After their invention, CA along with IEF became slowly commercialized. Today there are four major companies producing their own lines of CA: Ampholine (the original by Vesterberg patent), Bio-Lyte, Pharmalyte, and Servalyt. For detailed view of their properties see the review article[10]. One of the main requirements of a good ampholyte is to have a high buffering capacity in its isoelectric point (p*I*), which is fulfilled if individual pK_As difference is lower than two. Otherwise, the buffering capacity decreases with increasing gap between the pK_As[10].

1.2.2 Autofocusing

Surprisingly, there is an application where a complexity of sample matrix can be beneficial. On the contrary to commercially produced ampholyte mixtures, pH gradient can be sometimes formed by a sample itself[11,12]. Because proteins, peptides, and amino-acids are in great majority also

ampholytes, they can be used for creation of pH gradient. This approach was originally used before the ampholyte invention[13], during development of ampholyte mixtures[14], and recently for separation of tryptic peptides[15,16] and yeast extracts[17]. In recent works, autofocusing was used to avoid disadvantages connected with ampholyte mixture usage like mass spectrometry (MS) incompatibility[18], high ultraviolet (UV) absorption of CA below 260 nm, large CA volumes used in preparative scale analyses[19], or relatively high price[17]. Main drawback of this approach is nonlinearity of formed pH gradient. Therefore, there is a need for isoelectric point markers for gradient tracing[15–17].

1.2.3 IEF as a fractionation technique

There are several preparative scale IEF devices which are commercially available and each of them uses slightly different concept of IEF based on free-flow, solution-phase, off-gel, and in-gel approach. Individual modes of IEF possess various advantages and disadvantages which will be further discussed in ongoing paragraphs.

1.2.3.1 Free-flow IEF

In free-flow mode, analysis is performed in separation bed (chamber) which is continually flowed through by a solution (see Figure 1). Separation bed can be either flow through channel/chamber or it can be made from a porous matrix. Size of a separation bed varies from small systems in microchip formats to large volume systems capable to separate up to liters of a sample. Review articles are available for detailed insight into this technique[20–22]. Advantages of the technique are mainly short separation times and high sample throughput which makes it ideal for a fractionation of bulky samples.

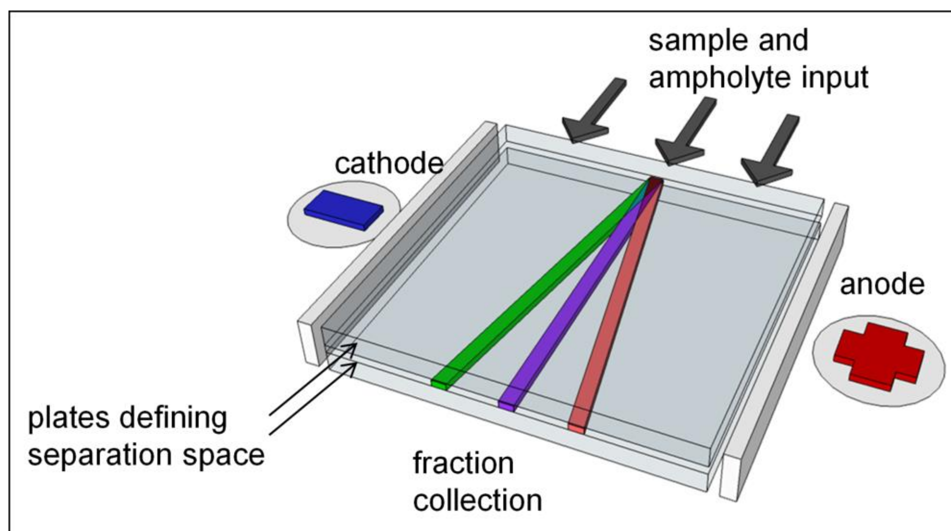

Figure 1 - 3D scheme of free flow IEF system

There have been many attempts to develop free-flow isoelectric focusing systems in microchip format[21,22]. In those systems gas bubbles produced in electrodes compartment may significantly affect analysis efficiency or they can even prevent separation itself. Various strategies were employed to deal with this problem. They include usage of open electrode chambers[23], usage of milled electrode chambers for bubbles trapping[24], separation of electrode chambers with microchannels[25], electrode microstructure covered by glass layer[26], and polyacrylamide gel segments[27–29]. On the contrary to rectangular shape of separation chamber in those microchip devices, divergent/triangular shape was suggested in more recent papers[30,31]. With this approach authors have been able to improve resolution, because separation occurred not only due to IEF but also due to flow geometry which helped to separate individual analytes. Further, Šlais[30] advantageously used separation bed made from layers of nonwoven fabric. This removed issues of gas bubbles formation during IEF fractionation and enabled to optimize separation space easily. Concerning biological matrices, free-flow IEF systems were used to separate:

proteins[15,17,24,26–28,32–37], peptides[15,35,38], colored/fluorescent isoelectric point markers[23,24,27–30,34,39], and nucleic acids[24]. Apart from mentioned laboratory-devised instruments, there is one system which is currently marketed – the BD™ Free Flow Electrophoresis (FFE) System. It is successor of the former Octopus FFE system. TECAN Company recently discontinued their ProTeam FFE system which was another successor of Elphor VAP system. Those systems are basically composed by two large glass plates with spacer between them which define shape and volume of a separating chamber. FFE Systems have high variability concerning several positions for sample and ampholyte solution introduction and maximum of 96 fractions can be collected after a separation[38,40–43].

1.2.3.2 Solution IEF

Conversely to the free-flow IEF, in solution IEF analysis is restricted into a medium and space of a separation chamber which can be optionally segregated into smaller parts divided by a semipermeable material. One of the first instruments based on IEF fractionation in free solution was Rotofor[44] which is currently commercialized by BioRad Laboratories. Focusing chamber of Rotofor is divided into compartments by membranes and electrodes are separated by ion-exchange membranes (see Figure 2A). Whole chamber is gently shaken during IEF and cooled by metal block clipped close to it. Range of pH gradient is determined by used ampholyte mixture. Recently it was used for a fractionation of proteins from human follicular fluid samples[45], tryptic peptides[15,16], and of small non-covalent metal species from plants[46]. Another microscale liquid phase IEF device is similar to the Rotofor but with a few exceptions. In the ZOOM® IEF fractionator from Invitrogen Corporation devised by Zuo and Speicher[47] membranes with defined pH (Zoom disks) are inserted between individual chambers (see Figure 2B). Whole cell lysates of *Escherichia coli* were

analyzed by this technique[48]. Akin instrument called membrane-separated wells for isoelectric focusing and trapping (MSWIFT) was presented by Gyula Vigh group[49]. Focusing chamber is divided by lab-made poly(vinyl alcohol)-based ion-permeable buffering membranes. Mode of IEF (e.g. desalting, fractionating, trapping) could be selected easily by usage of the right set and number of membranes[49]. This instrument was used for a separation of proteins and peptides followed by either LC-MS and MS[50,51]. Later on, a concept of a parallel IEF in microvolumes was invented by Zilberstein et al.[52] (see Figure 2C). Here a poly(methyl methacrylate) plate with set of drilled holes is used for fractionation. Each hole is filled with 2 µl of Immobiline buffer with acrylamide of different pK_A and, after filling all holes, Immobilines are polymerized. Then the plate is placed into a focusing chamber in parallel to the electrodes and IEF is performed. Analytes are focused in holes containing gel of appropriate p*K* and they can be extracted into the second electrophoretic dimension afterwards (e.g. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)). In this way, colored and colorless proteins were focused on this device [52–54]. The Gradiflow fractionating system is based on separation of sample into two fractions. Apart from p*I*, molecular size is also separating parameter in this device. Sample is recirculated in a cartridge which is divided into segments by a separation membrane. Furthermore, there are electrode compartments in the cartridge which are separated by restriction membranes and an electrophoretic buffer is flown through. As a result of a separation on this device, we can get two fractions of proteins differing in a pH and free from low-molecular size peptides[55]. Similarly based device called ConFrac contains anodic and cathodic flowed through chambers which are connected by set of trapping chambers bordered by membranes of defined pH. After an application of voltage

proteins migrate through trapping chambers until they found a chamber with pH corresponding to its p*I*[56]. An IEF fractionator based on focusing in nonwoven fabric strip which used simple buffers mixture for pH gradient formation, was described recently[57]. In this concept a nonwoven fabric strip placed into a tray and the fabric is loaded by sample mixed with simple buffers and ethylene glycol/glycerol. Proteins and peptides from dried bovine whey were separated using this technique[57]. Quite different approach was used in microscale isoelectric fractionation with photopolymerized membranes. Membranes of pH 5.3 and 6.5 were polymerized in microchannels by precisely directed UV light to control size and localization of membrane. The result of this technique was one fraction with proteins which had p*I*s within range of 5.3 – 6.5. Authors of this work emphasized easy recovery of focused analytes and capability to transfer focused species to subsequent analysis combined with the selective and scalable fabrication approach[58].

1.2.3.3 In-gel and off-gel IEF

IEF in gel media is well known to be the first dimension of the 2D electrophoresis gel approach. Since invention of immobilized pH gradient (IPG) in gel matrix[59,60] it has been very popular among scientists analyzing proteins in complicated matrices using 2D SDS-PAGE. Nevertheless, there were some attempts to use IPG IEF technique for fractionation of BM. After IEF analysis on IPG gel strip, strip can be cut into pieces and focused analytes extracted into fractions. For example proteins from a human blood plasma were separated in IPG strip which was followed by tryptic digestion of focused proteins and originated peptides from individual fractions were then analyzed using another IPG strip. Whole procedure resulted in 96 fractions of fractionated peptides. Authors identified more than 800 peptides using this concept[61]. Possible drawback of IEF in IPG is that

recovery of focused analytes is not an easy task. Therefore, some concepts facilitating recovery were suggested. One of them is OFFGEL system commercialized by Agilent technologies (see Figure 2D). The system is based on series of open chambers which are sealed at the bottom by IPG strip. Peptides and proteins are stimulated to migrate in IPG strip and from/into solution in chambers. Migration is stopped when analyte reaches chamber with its p*I* in range of pH of underlying IPG strip. Focused fractions are then easily extracted by sucking up liquid from individual chambers. This technique was demonstrated to be superior over the concept of integrated strong cation-exchange chromatography (SCX) with reversed phase liquid chromatography (RPLC) in the second dimension (MudPIT)[62] but in other work a high pH RPLC fractionation lead to identification of more peptides than SCX and OFFGEL fractionations[63]. IPG strip was also used in a different technique using segments of paper laid over IPG strip for an extraction of peptides from gel. Results from this paper-based IEF method (PIEF) indicate that it can outperform OFFGEL in acidic region whereas it is outperformed by OFFGEL in alkaline regions. Further, the authors evidenced about 500 unique peptides from each fractionation method and thus concluded that PIEF could complement OFFGEL[64]. There should be stated that most of the fractionation techniques using IPG strips suffer from long separation times which can spread from a few hours to 2 – 3 days[65].

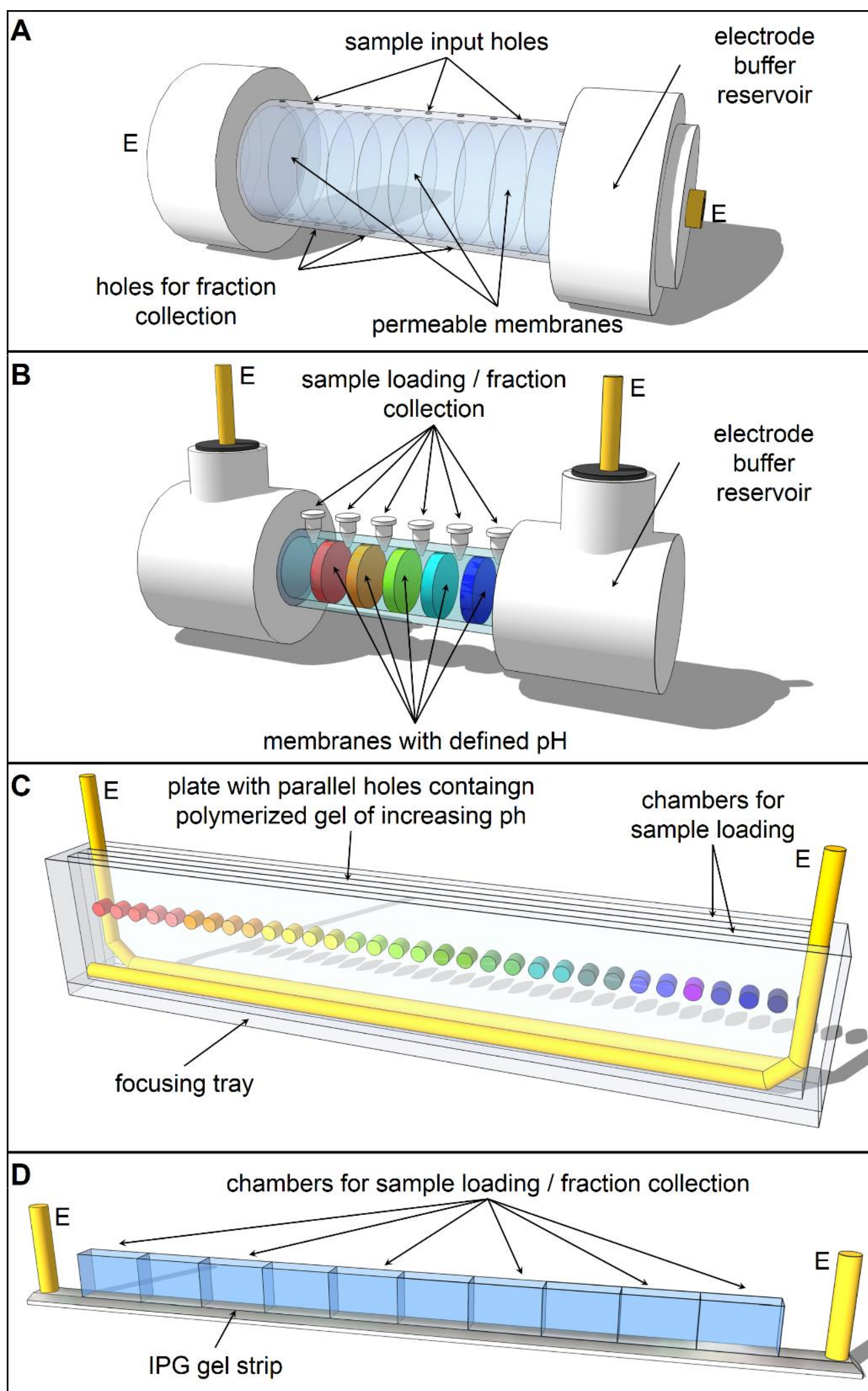

Figure 2 - 3D schemes of IEF fractionators; A - Rotofor device; B - ZOOM IEF fractionator; C - parallel IEF; D - OFFGEL; E - electrodes

1.2.4 Voltage, current and power in IEF

Power supplies are one of the most important devices considering IEF analysis. Resolution of a separation method is dependent on the applied voltage whereas, applied current is responsible for moving substances through a separation medium. Therefore, to obtain the best resolution/time ratio, IEF should be run under the highest power conditions available[66]. This principle is commonly applied in a planar gel and capillary format. Apart from those IEF modes, strip sized focusing devices are between those two size formats. Amount of total power, which can be applied without noticeable Joule heating, is there in range of tens of milliwatts. Such low wattage limit can be tricky because of current leakage and thus many of available electrophoretic power supplies do not have option to limit applied power below 1 watt. Therefore, it was recommended to limit current during IEF run with programmed voltage gradient[66]. One possible drawback of this approach is inevitable prolonging of analysis. Quite recently two companies have proposed their power supplies capable of IEF run with limits below 1 watt. Those state of the art power supplies are integrated solutions for IPG/OFFGEL IEF and due to their technology level they are quite expensive. As a low cost alternative to these sophisticated power supplies we have recently suggested a power supply based on the design of voltage multiplier.

1.3 Liquid chromatography

Throughout the last century, liquid chromatography evolved into a huge field of techniques which are used all over the world. Moreover, it is one of the most frequent techniques used for protein separations. Liquid chromatography is a part of classical approach in proteomics as a system composed from liquid chromatography – electrospray ionization (ESI) – tandem MS. Moreover, miniaturized nano-LC systems are now widely used due to increased sensitivity compared to analytical systems[67].

Therefore in last years, great effort was aimed to a miniaturization and increase in resolution of LC systems. Those attempts resulted in the development of ultrahigh pressure LC with sub-2 micron particles, monolithic columns with lowered backpressure, nano-LC systems and many more. Miniaturization was particularly important for analysis of complex mixtures due to increase in sensitivity if small amounts of sample were analyzed. In further paragraphs the most common LC modes will be described in regard to separation of proteins and peptides.

1.3.1 Reversed phase liquid chromatography

Reversed phase liquid chromatography is choice number one for most of the applications where very high resolution is needed. Analyte separation is based on its hydrophobicity in RPLC. Therefore, peptides and proteins are good candidates as many of them contain some hydrophobic regions in their structure. When optimized conditions were used, about 1000 proteins in concentration range 10^3 – 10^4 from *Shewanella oneidensis* could be identified during 50 minutes run[68] employing 50 µm internal diameter capillary column with submicrometer C18 particles. Moreover, one of beneficial features is RPLC capability to wash a polar matrix left in sample

from previous fractionation technique. This can be used for buffer exchange, desalting and concentrating of the proteins or peptides.

1.3.2 High pH reversed phase liquid chromatography

Although classical reversed phase approach is connected with acidic pH mainly due to utilization of trifluoroacetic acid or formic acid as ion pairing agents, high pH RPLC can also be used for fractionation. Even though the separation mechanism stays unchanged, charge of proteins and peptides is changed significantly by applying high pH and therefore elution order is also changed. It was reported that in terms of sample fractionation high pH RPLC is comparable to the SCX separation method[69] when followed by further RPLC analysis. It should be noted that special attention must be paid to a selection of a high pH stable RPLC column because common silica beads can be damaged by high pH. Thereby, only columns designated for such range of pH can be used (e.g. endcapped columns, columns with polymeric packing, or organic based monolithic stationary phase columns).

1.3.3 Strong cation-exchange liquid chromatography

Strong cation-exchange liquid chromatography is very popular tool for separation of proteins and peptides. In this separation mode, negative functional groups, which are embedded in a stationary phase, interact with positive charges of protein/peptide and analytes are exchanged with cations and therefore the most positively charged species are eluted last. Elution can be performed by increasing a salt concentration in a mobile phase (MF) where the retention of analytes is decreased due to increase in ionic strength and decrease in electrostatic interactions[65]. Another possible way how to elute analytes is change of analyte total net charge after change of pH above/below their p*I*. Both modes of the elution can be done using a step-wise increase or gradient of salt/pH in mobile phase. Although there

are also strong/weak anion exchange and weak cation exchange modes, SCX with the salt gradient is the most frequent separation employed in proteomics[65]. Tryptic peptides belong to a group of analytes which is especially suitable for fractionation with SCX. Based on their origin, most of those peptides have either lysine or arginine in their structure, therefore they have one positively charged amino acid residue regardless of their primary structure. In acidic conditions this results in two positive charges for peptides which do not have any other charged amino acids in their primary structure. However, it was found that peptides are eluted in clusters of the same charge when the salt gradient was used[69]. Apart from common fractionation, SCX can also be used for an enrichment of phosphopeptides thanks to their lower net charge compared to typical peptides originating during tryptic digestion[70]. The pH gradient elution is beneficial for coupling of SCX to MS detector because of its buffer compatibility. In case of salt elution, extensive washing steps are needed prior to MS detection[65]. However it was reported that many peptides can overlap throughout the fractions due to low resolution of SCX[71].

1.3.4 Hydrophilic interaction chromatography

Hydrophilic interaction chromatography (HILIC) is similar to normal phase liquid chromatography but separation mechanism in HILIC is more complicated[72]. In HILIC, analytes are separated according their hydrophilicity. The higher the hydrophilicity of analytes the higher the retention is. For elution are used mobile phases akin to reversed phase LC containing a high concentration of organic solvents and low concentration of water. As the concentration of water is increased, retention of a hydrophilic analyte decreases. Separation is based probably on partitioning of an analyte between a water layer absorbed onto a stationary phase and water contained in mobile phase although this was not proved

theoretically and some other interactions are expected to play a partial role in retention as well[72]. Compared to SCX, HILIC has mobile phase more compatible with MS. Moreover, due to increased hydrophilicity of phosphopeptides and glycopeptides, HILIC can be used for their fractionation[73–75].

1.3.5 Electrostatic repulsion hydrophobic interaction chromatography

Newly developed method called electrostatic repulsion hydrophobic interaction chromatography (ERLIC) is based on a stationary phase which has the same charge as analytes (stationary phases from SCX or strong anion exchange columns are often employed). Mobile phase is similar as in HILIC. Therefore retention is determined by electrostatic repulsion and hydrophilic interaction[76]. This method also became popular for analysis of phosphopeptides and glycopeptides as in the case of HILIC[76–78]. Moreover, it has been published recently, that ERLIC outperformed RPLC in analysis of complex mixtures of peptides[79].

1.3.6 Size exclusion chromatography

Size exclusion chromatography (SEC) is one of the techniques which are capable of separating sample according to *m* of particular analytes. Stationary phase has pores which can be entered by sample particles whereas large molecules cannot enter these pores. This results in slower migration of smaller molecules through the separation medium due to their retardation in pores and therefore larger molecules are eluted first. Depending on chosen column packing it is possible to separate molecules in range from 0.1 to 100 kDa[80,81]. Nevertheless, due to its lower resolution, SEC is more suitable for fractionation of proteins which can be further digested and separated with following technique[81]. However, necessary

calibration with appropriate standards, high amount of loaded sample, high volume of obtained fractions, and expensive columns are possible drawbacks of this method[81]. Other interesting approach is a mixed mode chromatography connecting SEC and SCX features together. Stationary phases for this method are called restricted access materials (RAMs). In RAM, analytes, which can enter pores, are trapped inside and they can be released by increasing salt concentration or by changing pH. For example, this separation technique was used for an online separation of human serum sample peptidome[82].

1.3.7 Affinity chromatography

Unlike chromatography methods mentioned above, affinity chromatography is based on specific biochemical interactions. It is used mainly for depletion and enrichment of selected proteins. Depletion is commonly employed to reduce a concentration range of proteins in a sample (e.g. in human serum plasma 55% of total protein is occupied by albumin[83] and the 22 most abundant proteins form 99% of total plasma protein[84]). Depletion methods are often based on avidity between a specific immunoglobulin and its target in a sample. Columns and cartridges containing various immunoglobulins for depletion of up to dozen of the most abundant proteins in specific samples are well established for protein concentration range reduction. However nonspecific binding can be a problem in depletion techniques which can result in a loss of proteins. This issue was not completely resolved, but there were suggestions that a non-specific binding of low abundance proteins to ligands and/or so-called “sponge effect” where small proteins bind to large ones which acts as carriers, may be the reason of codepletion[67].

On the other hand, enrichment is based on extraction of a particular group of analytes from a sample while discarding the rest. As a result, analytes of specific group are concentrated and therefore they are easier to detect. The most employed methods today include immobilized metal/metal oxide affinity chromatography for phosphopeptides enrichment[85] and lectin (proteins selectively recognizing glycan structures attached to glycoproteins) chromatography for glycopeptides enrichment[86].

1.3.8 Ligand library

Quite different approach to reducing protein concentration range is application of ligand library[87,88]. The word "library" implicates that it is composed from a large number of ligands. Millions of hexapeptides attached to polymeric beads are synthetized by combinatorial chemistry in this technique. Each bead carries a unique hexapeptide at a certain concentration of about 50 $mmol \cdot l^{-1}$ [67]. When sample is repeatedly washed through those beads, proteins are specifically bound to particular beads. When full capacity of individual bead is reached, excess of protein is washed away. Therefore, high abundance proteins are depleted while low abundant proteins are enriched. As a result, concentration of particular proteins is equalized, which can increase amount of protein identifications[89].

1.4 Two-dimensional methods

When the first two-dimensional techniques started to appear in the '40s there was still long way before they could be developed into sophisticated systems as we know them these days. The pioneer works dealt with 2D chromatography system, combination of chromatography and electrophoresis, and 2D system relying on two dimensions of electrophoresis only[90–92]. It took almost thirty years until the most popular 2D system in the protein research was discovered[93]. Combination of the isoelectric focusing in the first dimension with SDS-PAGE got many users through following decades thanks to its high resolving capacity and relatively simple instrumentation. For long time, it was the method number one but in recent days it slowly subsides to more sophisticated systems mostly including LC-MS combination[94]. This two-dimensional system enables separation utilizing chromatographic principles and following separation by mass over charge of ions. But still this system is not able to resolve more than 1400 – 1600 substances, which is predicted maximum of single dimension of RPLC[95]. Considering that biological sample may include thousands of proteins and when digested it can easily increase to tens of thousands of peptides, it is only logical that high resolving methods are further combined into multidimensional separations.

1.4.1 Peak capacity

Due to often usage of gradient elution with 2D LC methods, other descriptor than theoretical plates was needed for characterization of resolution. Therefore, performance is described by peak capacity (P). For such reasons, peak capacity is defined as the maximum number of peaks that can be theoretically separated on a column at given chromatography conditions. Peak capacity is calculated according to Equation 1 where t_g is

gradient (separation) time and *w* is peak width. Peak width for calculation is taken at 4σ (13.4% of peak height)[95]. Due to the fact that sample peaks usually do not use whole length of an elution gradient it should be shortened to portion of the gradient occupied by peaks of analyzed sample. Therefore, to get individual sample P, the length of the gradient should be measured from the first eluting peak to the last eluting peak[96].

$$P = 1 + \frac{t_g}{w}$$

Equation 1 – Peak capacity calculation

1.4.2 Orthogonality

When two or more separation methods are connected together, it is necessary to think about separation mechanism of each method. The aim should be to choose methods with the completely independent separation mechanisms. The ideal two-dimensional separation system would then have a peak capacity equaled to a product of the first and second dimension peak capacity. However, this definition is valid only when selectivities of separations are completely independent, and the entire 2D separation space is randomly populated by the peaks[97]. Moreover, this is not the case of the most frequently connected separation methods especially for the double LC systems. Therefore Gilar et al. investigated orthogonality of different chromatographic systems in the first dimension connected to RPLC as a second dimension[69,98]. They interestingly noted, that high pH RPLC had similar orthogonality to acidic pH RPLC as SCX[69].

1.4.3 Coupling of two separation systems

The two main ways of a coupling are employed in the two-dimensional systems: offline and online. In offline approach fractions from the first dimension are collected using either a collector device or manual handling.

A few advantages results from such process. Firstly, fractionated analytes can be stored for a subsequent analysis and moreover fractions can be analyzed repeatedly. Secondly, long separations can be applied in the second dimension and a sample fraction amount can be increased if necessary. Finally, there is place for a parallel processing of a sample. Main reproaches are possible sample losses during handling and storing of fractions and limited possibilities for analysis of low volumes of a sample. Online approach, on the contrary, is well suited for analysis of minute sample amounts and unstable compounds, and sample losses during fraction transfer are minimized. Main drawback include decreased flexibility in selection of second dimension, necessity to use compatible mobile phases and possibly use of expensive sophisticated systems[94]. Moreover, the coupling system is dependent on the first separation system used, due to problematic automation of fractions extraction from some systems (e.g. for solution-phase and gel IEF).

1.4.4 IEF offline coupled to RPLC

When it comes to multidimensional systems, IEF is often compared with SCX as a fractionation for RPLC second dimension, because analytes are separated according to the similar principle in those techniques. However, it is not clear from literature whether the one technique is superior over the other. With a IPG strip used for a fractionation of a peptide mixture coming from rat testis into 43 fraction Essander et al. got a higher number of identified peptides than with SCX approach[99]. Further, analysis of a cerebrospinal fluid with OFFGEL compared to SCX resulted in more protein identifications in favor of OFFGEL[100]. In another study OFFGEL was compared to an online MudPIT system (SCX and RPLC embodied into the one column) and results showed that both first dimensions were comparable in term of identified peptides/proteins[62]. Although similar

peptide identification were reached with both IPG IEF and SCX, IEF was reported superior over SCX due to high reproducibility and sufficient resolution[101]. Other work reported SCX superior over OFFGEL; however, the authors admitted that low amount of sample (100 μl) could be a reason[102]. Generally, using ultra narrow pH gradients such as 3.5 – 5, which is basically the p*I* range of most of the tryptic peptides, can produce more compelling IEF separation[103]. In addition, as RP stationary phase exhibited partial dependence on *m*, no correlation was found between IEF and *m* and therefore IEF and RPLC were reported to be orthogonal separations[38]. Recently, three-dimensional separation system comprised from IEF-SCX-RPLC was suggested for analysis of very complex mixtures[104].

Considering less common connections, IEF fractionation in IPG was compared to 1D SDS-PAGE, 1D preparative PAGE, and 2D SDS-PAGE as a fractionation for RPLC in the second dimension and even though IEF proved to identify high number of unique proteins an approach of combined utilization of IEF and 1D SDS-PAGE fractionation was suggested for proteomic profiling experiments[105]. Recently, high pH RPLC was found superior over OFFGEL IEF identifying 17% more proteins[106]. Further IEF-RPLC system was used for study of p*I* and retention time shift as a result of post-translational modifications[107]. SDS-PAGE proved to be the best technique for a characterization of the yeast nuclear proteome in study where SCX, IPG IEF, phosphocellulose P11 SDS-PAGE, and SDS-PAGE were used for fractionation prior to RPLC[108]. Free-flow IEF coupled to RPLC was suggested as a promising technique for proteomic analysis of colorectal cancer samples separating low *m* proteins[109]. In the beginning of proteomic era, rotofor IEF and 2D SDS-PAGE were used for fractionation of cellular proteins prior to nonporous RPLC, where

IEF fractionation doubled the number of identifications of proteins compared to 2D SDS-PAGE [110]. Similar system was then used for a creation of an ovarian carcinoma cell protein mass map and search for candidate cancer biomarkers respectively [111,112]. Proteins were fractionated with Rotofor which was followed by tryptic digestion and RPLC to demonstrate the described process as an alternative technique to other approaches used in proteomics[113]. System including Rotofor IEF fractionation with tryptic digestion of proteins fractions was developed to prevent protein losses in ongoing RPLC separation due to an extreme hydrophobicity/hydrophilicity of some proteins[114]. A general protocol for two-dimensional separations of intact proteins from whole-cell lysates using Rotofor device followed by RPLC was also published[115]. ^{18}O labeled peptides were fractionated by a solution phase IEF containing 6 chambers separated by membranes with defined pH and fractionated peptides were further separated by RPLC and identified using MS. Uncertain correlation between theoretical p*I*s and observed p*I*s of proteins in basic pH was discussed for the system[116]. A similar device containing membranes with a defined pH which divided the focusing chamber was used for an accurate comparison of protein expression between different bacterial samples[117]. Capillary IEF was offline coupled to RPLC by collecting fractions into individual wells on a microtiter plate with a modified HPLC sample collector. High voltage circuit was connected by immersing one end of a capillary in anolyte while coaxial sheath flow of catholyte was applied to the sampling end[118]. This approach was used for a comparative proteomic study of ovarian endometrioid adenocarcinoma cells[119].

1.4.5 IEF online coupled to RPLC

Capillary IEF (CIEF) is well suited for online coupling to RPLC thanks to low volume and simple transfer of fractions. Moreover, its high focusing

resolution and concentrating effect are also beneficial in multidimensional systems[120]. Important aspect of connecting CIEF into other separation method is necessity of HV decoupling. Various approaches were used including a hollow fibre membrane interface[121], cellulose acetate membrane coated capillary fracture[122], microdialysis membrane-based cathodic cell[123], and etched porous interface[124]. Different approach using microinjection valve connected to set of trap RPLC columns was used for 2D CIEF-RPLC analysis of proteins and peptides[125–129].

Unusual system composed of RPLC in the first dimension and CIEF in the second dimension was used for separation of model proteins and rat liver tissue extract. HV was switched off right after focusing and fractions were deposited on a plate for matrix assisted laser desorption/ionization by pressure mobilization[130]. RPLC fractionated sample was analyzed using CIEF with a sheath flow catholyte liquid and the liquid was subsequently changed to perform electrochemical mobilization into ESI-MS[131]. RPLC fractionation was also used for proteins from a cancer liver tissue extract which were further separated by an array of up to 60 CIEF capillaries with laser induced fluorescence whole column detection[132].

1.4.6 IEF connected to other techniques than LC

Online system consisting of CIEF with a capillary electrochromatography (CEC) as the second dimension was used for an analysis of standard peptides and proteins and a human serum sample. Decoupling of HV between individual dimensions was realized by switching a 6-way valve[133]. Cellulose acetate coated fracture in a capillary was used for decoupling of HV in CIEF-CEC system analyzing standard peptides and peptides from human red blood cells[122]. Decoupling of CIEF before further molecular mass-based separation by field-flow fractionation in a hollow fiber was

realized by 10 kDa cutoff membrane for separation of intact proteins[134]. Online connection to capillary isotachophoresis, which was realized by a microdialysis junction, was used for separation of tryptic peptides from standard proteins[135]. MSWIFT instrument was used in 2D approach employing capillary zone electrophoresis (CZE) as the second dimension. Moreover, the authors declared that obtaining information about p*I* from the first dimension contributed to better protein identification[136]. System consisting of CIEF online coupled to CZE was employed for an analysis of two fluorescently labeled proteins and a fluorescent p*I* marker and peak capacity of 125 was reported[137]. CIEF with immobilized pH gradient on the capillary wall which was used in 2D system prior to CZE managed to resolve proteins from cow's milk[138]. System connecting CIEF with CZE was capable to produce five fractions from the first dimension in which hemoglobin and myoglobin variants were separated[124]. The same group lately proposed CIEF coupled to capillary nongel sieving electrophoresis and the system was tested by separation of proteins excreting from lung cancer cells[139]. A mixture of tryptic peptides from four proteins was fractionated using OFFGEL and further analyzed by CZE. Authors found peak capacity to be roughly 700 and proposed this method as an alternative to IEF-RPLC approach[140]. Theoretical peak capacity of 24 000 was reached by analysis of tryptic peptides from bovine serum albumin (BSA) using system with online coupled CIEF and CEC[122]. Earlier works concerning coupling CIEF with other techniques were summarized in review by Kilár[141].

1.5 Whey

Milk (if not stated otherwise bovine milk is meant) is regarded and highly appreciated as an easily accessible source of proteins, peptides and calcium. Moreover, milk is processed into a number of products including cheese, yoghurt and other derivatives. During cheese manufacturing whey is produced as a side-product and is usually dried to be further used as a food additive or as a basis for processing of protein supplements.

1.5.1 Whey composition

Composition of the whey in connection to human health and diet is discussed in the following text. Whey originates during cheese producing when cheese proteins began to precipitate and recess solution is expelled away. Therefore whey contains all soluble substances from milk which do not precipitate into a cheese curd. Whey is divided into two types depending on a process used for cheese-making. When milk is treated by chymosin which cleaves casein (80% of total milk protein occupied by casein) specifically, then resulting whey is called sweet whey. On the contrary, when acidophilic bacteria are used for protein cleavage, whey is termed as an acid whey. Produced whey contains soluble proteins and peptides resulting from this cleavage which possess distinct health effects (approximately 20% of total milk protein is transferred to whey)[142]. The most abundant proteins in whey are α-Lactalbumin, β-Lactoglobulin, and caseinomacropeptide (CMP). From this three substances caseinomacropeptide have received much attention thanks to its biological activity[143] and will be described hereafter.

1.5.2 Caseinomacropeptide

CMP originates during cleavage of κ-casein by chymosin (or pepsin) between Phe105 and Met106 residues. CMP is formed by 64 amino acids

from C-terminal and it is sometimes called glycomacropeptide in literature[144] with overall molecular mass of 6.7 kDa (considering amino acid backbone only)[145]. Moreover, up to 11 genetic variants of κ-casein are known, but two genetic variants of CMP (A and B) dominate in bovine milk [143,146]. Those genetic variants differ in two amino acids (A: 136Thr, 148Asp; B: 136Ile, 148Ala).

1.5.2.1 Glycosylation of caseinomacropeptide

The second name suggests that CMP may be extensively glycosylated. Moreover, glycosylated forms represent about 50% of total amount of bovine CMP. N-acetylneuraminyl, galactosyl, and N-acetylgalactosamine units form up to tetrasaccharides attached to CMP amino acid backbone[147]. Glycosylated CMP have molecular mass in range from 7 to 11 kDa depending on a degree of glycosylation[148]. Notably, apparent molecular mass detected by gel filtration ranged from 20 to 50 kDa at pH 7.0 and from 10 to 30 kDa at pH 3.5[149–152]. The pH-dependent mechanism of apparent molecular mass changes is not still resolved. Xu et al. suggested that CMP could associate itself to form oligomers at neutral pH through non-covalent interactions, which partially dissociate at acidic pH[151]. Isoelectric point is also dependent on a degree of glycosylation (pK_A of sialic acid is 2.2) and therefore it ranges from 3.15 for fully glycosylated CMP to 4.15 for CMP form without glycosyls (aglycoCMP)[148].

1.5.2.2 Health effects of caseinomacropeptide

One of the health benefits of CMP is high amount of branched-chain amino acids and low amount of Met which makes CMP suitable for diet of people suffering from hepatic diseases[153]. Due to absent phenylalanine in CMP structure, it is possible to use CMP as a dietary supplement in nutrition of people suffering from phenylketonuria[154–156]. Moreover, high content of

sialic acid in carbohydrate chains could be beneficial for brain condition due to its incorporation into gangliosides and glycoproteins in brain. This was suggested by experiments with laboratory animals where exogenous administration of sialic acid and CMP improved learning ability[143,157,158]. Carbohydrate chains are important for further health effects including inhibition of the binding of cholera toxins to their oligosaccharide receptors on cell walls, protection from infection by influenza virus, inhibition of adhesion cariogenic bacteria to the oral cavity, and modulation of dental plaque microbiota[143].

1.5.2.3 Caseinomacropeptide as a food additive

Apart from health effects, CMP exhibits some physicochemical properties which can be used in development of novel foods or change of their rheological properties with possible health promoting effect. CMP was reported to have a good emulsifying capacity, but the capacity decreases after 24 hour of storing. Moreover, approximately 10% CMP solution is capable of a gel formation. Finally, CMP can create foam which has higher overrun but lower stability than egg white[143].

1.5.2.4 Purification of caseinomacropeptide

Thanks to described properties separation and characterization of CMP can become tricky. Recent techniques for production/purification of CMP mostly include chromatography and ultrafiltration. SCX was usually used for purification but also strong anion exchange (SAX), SEC, and HILIC usage were reported. Ultrafiltration membranes in range from 10 to 50 kDa were employed to separate CMP from α-Lactalbumin and β-Lactoglobulin. Because absorption at 280 nm is negligible thanks to absence of Phe, this parameter is commonly used for determination of CMP purity.

Analysis of CMP purity is commonly performed by HPLC and electrophoretic methods.

1.5.2.5 Separation of caseinomacropeptide

Modes used for separation were RPLC[144,159–166], SCX[151,160] and SAX[167–170]. When RLPC separation is employed, it is possible to separate CMP into clusters eluting from the least retained glycosylated CMP variant A and B peaks, then peak of aglycoCMP A, peaks of monoglycosylated CMP variant A and finally peak of aglycoCMP variant B. Moreover, electrophoretic methods were used for CMP separation including 1D SDS-PAGE[160,171,172] CZE[166,173], and CIEF[174] However, due to CMP heterogeneity it is difficult to resolve all variants in only one separation dimension[143]. Therefore, devising of a further separation method with a higher resolving capacity would be beneficial.

2 RESEARCH RATIONALE AND AIMS

Analysis of complex mixtures is not an easy task considering their heterogeneity. Therefore development of new methods which are able to separate and purify particular substances is highly important. Since biological samples can contain several thousands of individual substances, it is necessary to apply more separation methods in series for a separation of sampled mixture into peaks/zones containing only one or a few analytes.

This dissertation was elaborated at the department which is concerned with the development of advanced separation methods based on either isoelectric focusing or micro/nano liquid chromatography. Main aim of my thesis was to include those methods into two-dimensional schemes and employ this integrated approaches for separations of mixtures of standard proteins and peptides and biological mixtures.

Divergent-flow IEF is usable mainly for fractionation of voluminous and low concentrated samples where continual mode along with high concentrating and desalting effect are very beneficial. Mixture of tryptic peptides from BSA, myoglobin, and cytochrome c is good standard which can be used for testing of free-flow IEF device. Already established fractionation batch IEF methods were reported to be efficient tools for fractionation of proteins and peptides and therefore, their comparison with newly developed ones could be advantageous. Hence, I considered development of two-dimensional approach including divergent-flow IEF with capillary RPLC for analysis of real sample containing proteins and peptides as coherent with the main aim.

Common problem of solution IEF fractionation techniques is difficult and/or incomplete fraction extraction after the focusing was done.

Moreover, sometimes consumable parts used for analysis can be quite costly (e.g. IPG strips) or preparation of needed parts can be time consuming (e.g. fabrication of membranes with defined pH). Thus, I proposed a design of analysis using solution IEF based on nonwoven fabric separation bed excluding mentioned drawbacks and it was used for fractionation of whey before further analysis by RPLC.

RPLC is often used as a 'black box' without many possibilities for modification or coupling with further separation dimensions. Thereby, the next aim of this thesis was to develop and evaluate computer controlled simple chromatographic system based on the previously published concept of splitless formation of gradient of mobile phase in one syringe which would enable easy coupling to the first dimension.

Current fractionation techniques using strip format suffer from a long time needed for analysis. This is mainly due to a power load limit which can be applied to separation bed without production of excessive Joule heat. Only expensive state of the art IEF power supplies are able to limit a power directly. The rest is dependent on a combination of voltage and current limitation. The last aim of the thesis was therefore to construct a power supply which could limit the power applied to a separation bed using features of a voltage multiplier and evaluate it for the use in IEF analysis.

3 RESULTS

3.1 Research article I

Divergent-flow isoelectric focusing for separation and preparative analysis of peptides

Proteins are class of analytes well known for their easy focusing in IEF methods. On the contrary, peptides may be affected by a low surface net charge and/or electrical neutrality over a wider pH region compared to proteins. This is caused by the lack of charged residues in common peptide primary structure and it may result in poorer focusing compared to proteins. Divergent-flow isoelectric focusing device (DF-IEF) has been introduced in 2008 by Šlais[30] as an efficient and economical technique for fractionation with free-flow IEF. Since then the technique was employed for analysis of proteins[175], yeast lysates[17] and components in beer[37]. Therefore, peptide fractionation was a next step for characterization of DF-IEF device fractionation potential. Autofocusing of tryptic peptides mixture originating from BSA, cytochrome c, and myoglobin was tested to found whether the technique could be run without carrier ampholytes in sample mixture. This could prevent issues connected to CA usage (see section 1.2.2.) Obtained results were compared to Microrotofor device due to its established position in analysis of peptides. The resolving power of both techniques was then examined using either RPLC with ultraviolet wavelength diode array detector (UV DAD) or ESI-MS. MS data were subsequently used to identify number of peptides in individual fractions from DF-IEF.

Furthermore, DF-IEF was employed for purification of CMP from dried bovine sweet whey. Whey was used due to its complexity and availability and successful analysis could prove if DF-IEF device is capable to purify

particular substances from mixture without using external carrier ampholytes. RPLC with detection at wavelengths 214 nm and 280 nm was used for confirmation of CMP separation. It should be noted that focusing efficiency was so high that we observed concentration loss of two main protein components from whey sample due to their precipitation in separation bed. This was unexpected due to low demands for fractionation resolution as we collected only 12 fractions. As a result of whey fractionation, we suggested that DF-IEF is suitable for analysis of real samples and can be used for purification of CMP.

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Research Article

Divergent-flow isoelectric focusing for separation and preparative analysis of peptides

A divergent-flow isoelectric focusing (DF IEF) technique has been applied for the separation and preparative analysis of peptides. The parameters of the developed DF IEF device such as dimension and shape of the separation bed, selection of nonwoven material of the channel, and separation conditions were optimized. The DF IEF device was tested by the separation of a peptide mixture originating from the tryptic digestion of BSA, cytochrome c, and myoglobin. The pH gradient of DF IEF was created by the autofocusing of tryptic peptides themselves without any addition of carrier ampholytes. The focusing process was monitored visually using colored p*I* markers, and the obtained fractions were analyzed by RP-HPLC and ESI/TOF-MS. DF IEF operating in the autofocusing mode provides an efficient preseparation of peptides, which is comparable with a commercially available MicroRotofor multicompartment electrolyzer and significantly improves sequence coverage of analyzed proteins. The potential of the DF IEF device as an efficient tool for the preparative scale separations was demonstrated by the isolation of caseinomacropeptide (CMP) from a crude whey solution.

Keywords:
Autofocusing / Caseinomacropeptide / Isoelectric focusing / Mass spectrometry / Peptides
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1 Introduction

Biological samples represented mainly by peptide and protein mixtures are usually very complex and their separation/fractionation plays a key role in the identification of individual components [1]. Fractionation of peptide/protein samples prior to MS analysis minimizes the ionization suppression and significantly improves their identification [2, 3]. Highly efficient separations of peptides can be obtained by multidimensional liquid chromatography combining mainly strong cation-exchange and reversed-phase separation mechanisms with tandem mass spectrometric analysis [4]. On the other hand, electrophoretic separations such as 1D- or 2D-PAGE are still routinely used techniques in proteomic research [5]. However, these techniques are relatively time consuming and require expensive instrumentation.

Isoelectric focusing (IEF) in different free-solution formats [6–12] has been employed for the separation of peptide and protein mixtures as an alternative method to chromatographic and gel-based electrophoretic methods. Since the first proposal of electrophoretic separation in a free zone published by Hjerten in late 1960s [13], a variety of devices based on IEF have been developed for the protein and peptide fractionations. Some of them have already been made commercially available in both, analytical and miniaturized formats. An overview of these techniques including multicompartment and continuous-flow devices can be found in a review article published by Righetti et al. [14]. However, there has been a lack of new IEF techniques and devices suitable for the separation of peptides. In contrast to proteins with well-defined isoelectric points (p*I*) [15], the IEF separation of peptides is very often negatively affected by a very small net charge or electrically neutral nature of peptides over a broad range of pH. Thus, only peptides containing sufficient number of ionizable groups can be successfully separated [16]. A special attention has been paid to the capillary IEF technique that allows coupling with mass spectrometry [17] or capillary electrochromatography [18]. IEF separation of peptides has also been performed in a microchip format achieving ultrasensitive analysis of the minute amount of samples [19, 20]. On the other hand, commercially available instruments such as a preparative multicompartment electrolyzer are able to process larger sample volumes of up to tens of milliliters. For example, fractionation of peptides originated from tryptic digests of casein, lysozyme, and ovalbumin [21] or β-lactoglobulin [22] has been described. The miniaturized version of the multicompartment electrolyzer has been applied

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Abbreviations: **CMP**, caseinomacropeptide; **DF IEF**, divergent-flow isoelectric focusing

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to the preseparation of tryptic digests of casein, cytochrome c, myoglobin, and BSA, followed by the offline analysis of individual fractions by CZE and ESI/TOF-MS [23].

Due to the amphoteric character of peptides that enables to establish a pH gradient, the mentioned IEF experiments have been carried out in an autofocusing mode in the absence of carrier ampholytes. The autofocusing mode eliminates the problems associated with the presence of carrier ampholytes in IEF fractions, which are usually subjected to subsequent analyses by HPLC and/or MS [24]. Off-Gel IEF of proteins and peptides has also been investigated [25] and successfully coupled with RP-HPLC/MS [26, 27]. Although the separation is performed in a liquid chamber, this technique is not purely liquid based due to the presence of an IPG gel creating the pH gradient in the separation chamber. Furthermore, proteins and peptides can remain in the gel and the extraction step is needed to enhance their yield [26]. Despite the construction of high volume (up to 50 L) multicompartment electrolyzers dedicated to industrial applications [28], the volume capacity of a processed sample is often limited. The IEF fractionation using the multicompartment or Off-Gel IEF devices requires long operation time varying from few hours to several days.

Free-flow electrophoresis (FFE) described by Hannig [29] overcomes the limitations associated with operations in a batch mode. FFE offers several advantages including a solution phase separation mode without any need for further extraction from the gel matrix, large loading capacities and flexibility of loading volumes, high recoveries, rapid separation, and easy collection of sample fractions. FFE-IEF fractionation of cellular proteins and peptides followed by RP-HPLC analysis has been described by Moritz et al. [8]. FFE-IEF has been proven as a high-resolution method for peptide separation and determination of their isoelectric points. Due to the determined p*I* values of peptides, the improved accuracy of sequence database searching and followed protein identification was demonstrated by analysis of a chromatin-enriched fraction from the yeast *Saccharomyces cerevisiae* and a cytosolic fraction from Schneider S2 cells of *Drosophila melanogaster* [9, 30].

Recently, a new concept of FFE-IEF called divergent-flow IEF (DF IEF) has been suggested by our group [31]. In this technique, a planar divergent flow of medium was subjected to transversal IEF in a continuously widening flat channel made from a nonwoven material, in which the liquid flows from the narrow channel input toward the wide output. DF IEF has been successfully applied to the separations of colored low-molecular-weight p*I* markers [32], standard proteins such as hemoglobin and cytochrome c [33] as well as for the fractionation and purification of crude protein extracts from barley grain, malt, and beer [34]. Furthermore, the separations of protein extracts isolated from complex biological samples (e.g. yeast cell lysate, wheat flour extract) were demonstrated using the DF IEF device operating in the autofocusing mode [31].

The aim of this work was an optimization of the DF IEF device with respect to the dimension and shape of the separation bed, selection of a nonwoven material of the separation channel, and conditions for the fractionation of peptides. A complex sample consisting tryptic digests of BSA, myoglobin, and cytochrome c was used as a model peptide mixture and the individual fractions were analyzed by RP-HPLC and mass spectrometry. The ability of the developed DF IEF device to operate in a preparative mode was demonstrated by the isolation of caseinomacropeptide (CMP) from a solution of crude whey.

2 Materials and methods

2.1 Chemicals and materials

L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) treated bovine trypsin, BSA, bovine cytochrome c, equine myoglobin, bovine α-lactalbumin, bovine β-lactoglobulin, bovine CMP, DTT, iodoacetic acid, ACN, TFA, sodium hydroxide, potassium hydroxide, potassium sulfate, sulfuric acid, hydrochloric acid, phosphoric acid, and Tris were purchased from Sigma-Aldrich (Prague, Czech Republic). The nonwoven polypropylene-based PEGAS-AGRO fabric was purchased from PEGAS NONWOVENS (Znojmo, Czech Republic).

2.2 Preparation of peptide mixture

A peptide mixture was prepared from the tryptic digests of BSA, myoglobin, and cytochrome c. Each protein was digested separately. BSA (5 mg) was dissolved in 1.25 mL solution of 100 mmol/L Tris containing 50 mmol/L DTT and disulfide reduction was performed at 70°C for 10 min. After cooling to the room temperature, BSA was alkylated with 2.5 mL of 100 mmol/L iodoacetic acid prepared in 100 mmol/L Tris solution for 30 min at room temperature. Before the digestion, salts were removed using precipitation of alkylated BSA by addition of 1 mL of 5% (v/v) TFA and incubation for 30 min at −20°C. The precipitate was separated by centrifugation, washed twice with 750 μL of 5% (v/v) TFA, and finally dissolved in 1 mL of 50 mmol/L ammonium bicarbonate solution pH 8.0. Cytochrome c and myoglobin (without any previous treatment) were dissolved in 1 mL of 50 mmol/L ammonium bicarbonate solution pH 8.0 to obtain a solution with a concentration of 1 mg/mL. Trypsin was added at a substrate-to-enzyme ratio of 50:1 (w/w) and the solutions were incubated at 37°C for 16 h. The tryptic digest solutions were divided in the vials and vacuum dried, and the pellets were stored at −20°C before further use.

Before peptide focusing using the DF IEF device or the MicroRotofor multicompartment electrolyzer, the pellets of BSA (1 mg), cytochrome c (0.5 mg), and myoglobin (0.5 mg) tryptic digests were redissolved in 50 mL of water. A 10 mL portion was used for fractionation using the MicroRotofor multicompartment electrolyzer. A 40 mL portion of protein digest solution was applied to DF IEF.

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2.4 Peptide focusing using a DF IEF device

A basic device setup has been described previously [31, 32] and its modification for this study is discussed in Section 3. The IEF separation of tryptic digests and a crude whey solution was performed in the autofocusing mode without the addition of carrier ampholytes. Sodium hydroxide (5 mg), potassium hydroxide (2.6 mg), and potassium sulfate (30.2 mg) were added to the 40 mL portion of the protein digest solution. Whey powder was dissolved in deionized water to obtain a solution with a concentration of 10 mg/mL. A mixture of synthetic low-molecular-weight pI markers synthesized in our lab [35–37] was used to monitor the pI gradient formation.

Due to the limited amount of the sample, the separation of the tryptic digests was carried out using a 2-pair electrode DF IEF device; however, the separation of whey was performed using a 3-pair electrode DF IEF device. The separation/focusing bed was equilibrated with a separation medium containing 3.15 mmol/L sodium hydroxide, 1.17 mmol/L potassium hydroxide, and 4.34 mmol/L potassium sulfate for 10 min and then peptide mixture or whey solution, respectively, was loaded to the separation channel. The separation conditions were set as follows: voltage: 550 V, current: 8 mA, and power load: 4 W. The limit of 10 W was set for separation of whey. The flow rate was maintained at 160 or 190 μL/min, for separation of protein digests and crude whey, respectively. Separations were performed at 6°C in the refrigerator. The separated fractions were collected into 12 vials and kept at −20°C before further analysis.

2.5 Peptide focusing using a MicroRotofor multicompartment electrolyzer

The MicroRotofor multicompartment electrolyzer equipped with the PowerPac 3000 power supply (BioRad, Hercules, CA, USA) was used for IEF of peptides in the autofocusing mode [23]. The ion exchange membranes, separating the electrode reservoirs and the focusing chamber, were equilibrated in 0.1 mol/L H_3PO_4 (cation exchange membrane) and 0.1 mol/L NaOH (anion exchange membrane), respectively. The mixture of tryptic digests was injected into the focusing chamber by a 3 mL syringe until all ten compartments were equally loaded. Both the loading and harvesting holes on the opposite sides of the focusing compartments were sealed by an adhesive tape. Focusing assembly was positioned in the cooling block and the oscillating motor gently rocking the focusing assembly was turned on. The voltage program was used with the start at 50 V (30 min) and gradually increasing to 400 V until reaching a power limit of 1 W. The run was terminated after about 2 h, when the current stopped decreasing (the current value leveled around 1 mA). After focusing, ten fractions were transferred into vials and kept at −20°C before further analysis.

2.6 Liquid chromatography

All chromatographic experiments were performed using an Agilent 1200 Series chromatographic system (Agilent Technologies, Santa Clara, CA, USA). Separations were performed on a microbore Poroshell 300SB-C18 column (5 μm particle size, 1 × 75 mm, Agilent Technologies) equipped with a C18 cartridge guard. The elution was run at a flow rate of 20 μL/min and 70°C by a binary gradient with ACN as an organic modifier. Solvent A consisted of 0.1% (v/v) TFA in water and solvent B consisted of 0.1% (v/v) TFA in ACN. Linear gradients from 5 to 35% (v/v) solvent B over 12 min and from 5 to 80% (v/v) solvent B over 30 min were used for separation of tryptic digests and whey, respectively (gradient dead time: 5 min). The detection was performed at 214 nm and 280 nm using a diode-array detector.

2.7 Mass spectrometry

The untreated mixture of tryptic digests and collected fractions were desalted using a C18 ZipTip (Millipore, Billerica, MA, USA) following the protocol suggested by the manufacturer. The Mariner electrospray TOF mass spectrometer–ESI/TOF (ABI, Framingham, MA, USA) was used in the positive ion mode in the range of 400–1500 *m/z*. MS infusion analysis was conducted using a nanospray prepared from 10 μm id (365 μm od) fused-silica capillary with a polished tip. The list of detected ions (*m/z*) was used for peptide and protein identification by MS-Fit peptide mass fingerprinting tool of Protein prospector database (http://prospector.ucsf.edu).

3 Results and discussion

3.1 DF IEF device

The design of the DF IEF device developed in our laboratory [31] was modified in order to obtain an efficient separation of peptides (Fig. 1A). Due to the low concentration of peptides and negligible content of salts in tryptic digests, a length of the separation bed was shortened to a half, and the middle pair of electrodes was removed in comparison to the previously used design. The modified DF IEF device was applied to the separation of tryptic digests originated from BSA, cytochrome c, and myoglobin, and followed by RP-HPLC and mass spectrometric analyses.

Several materials for creating the separation bed of the DF IEF device have been used in the previous studies. For example, hydrophilized polypropylene-based Pegatex S 17 (PEGAS NONWOVENS) and polyester-based Vlieseline (Freudenberg Vliesstoffe, Weinheim, Germany) nonwoven fabrics were successfully utilized for the DF IEF separation. These nonwoven fabrics have a very good mechanical stability and high permeability, and the efficient separations of proteins and cell lysates were obtained. However, these materials were found to be unsuitable for the subsequent ESI/MS analysis due to the sample contamination with synthetic

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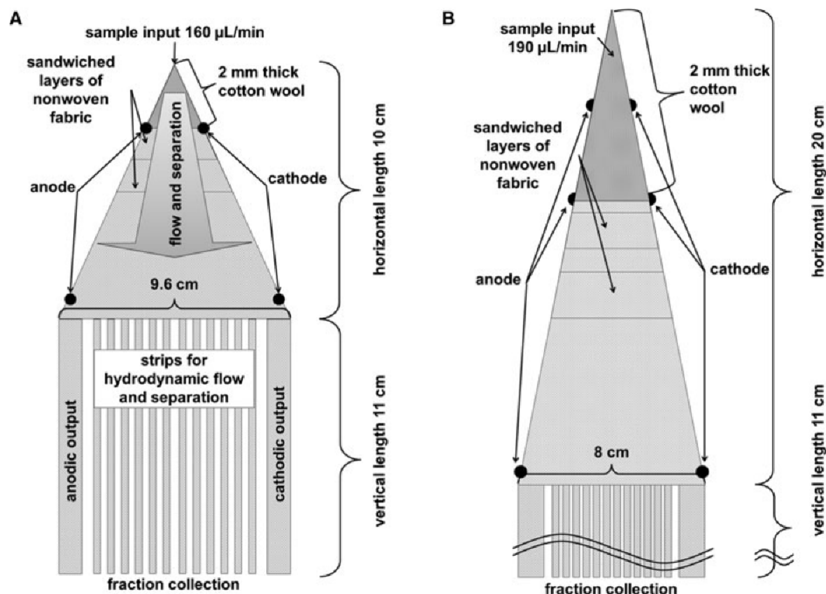

Figure 1. Design of DF IEF devices used for preseparation of peptide mixture (A) and isolation of caseinomacropeptide from whey solution (B).

polymers. In the preliminary experiments, small pieces of fabrics were immersed into the aqueous solutions of BSA digest for 30 min that were subsequently analyzed by ESI/TOF-MS. The series of peaks separated by 44 Da corresponding to the mass of the ethoxylated groups dominated in the mass spectra with only a few low intensive peaks corresponding to the peptide fragments. Apparently, these materials forming the separation channel were modified with polyethylene glycol during the industrial manufacturing in order to make these materials more hydrophilic. After testing of several materials, we chose a polypropylene-based nonwoven fabric PEGAS-AGRO (PEGAS NONWOVENS) without any hydrophilization treatment. We did not observe any contamination of analyzed BSA digests by ESI/TOF-MS analysis and the mass spectra contained only peaks corresponding to the tryptic fragments of BSA.

3.2 Separation of tryptic digests

The aim of this study was to demonstrate the effectiveness of the DF IEF device as a tool for peptide separation. In order to minimize the complexity of the sample prior to MS detection, DF IEF was performed in the autofocusing mode with peptides serving as a pH gradient medium. The mixture of peptides was introduced into the focusing bed using a peristaltic pump and focused on the bases of p*I*s. The focusing process was monitored visually using a mixture of colored p*I* markers with the isoelectric points of 3.0 (orange), 5.3 (purple), 6.2 (red), 8.5 (yellow), and 11.0 (purple) synthesized in our lab [35–37]. Individual markers were added to the BSA (0.02 mg/mL) digest in the presence or absence of carrier ampholytes. After 30 min, the p*I* markers were well focused in lines according to their isoelectric points. Twelve fractions were collected (Fig. 2A), where fraction 1 represented the most acidic fraction and fraction 12 the most alkaline one in the set. The individual fractions colored by separated p*I* markers demonstrated the efficient autofocusing effect of the peptide mixture. For comparison, the same BSA digest was also analyzed in the presence of carrier ampholytes (0.1 mL carrier ampholytes/25 mL) [32, 34, 35, 37] with similar results (Fig. 2A). Figure 2B shows a plot of the pH values measured in the individual collected fractions. As expected, a smoother pH distribution in the carrier ampholyte containing fractions was observed in contrast to a wavy distribution in the case of autofocusing.

The effectiveness of fractionation was evaluated by the separation of the peptide mixture originating from tryptic digestion of several proteins covering a range of p*I*s from 5.6 (BSA), 7.4 (horse myoglobin), to 9.5 (bovine cytochrome c). After separation using the DF IEF device, the contents of individual fractions were analyzed without further treatment by RP-HPLC with UV detection at 214 nm. Figure 3 shows chromatograms of the original mixture and all 12 fractions

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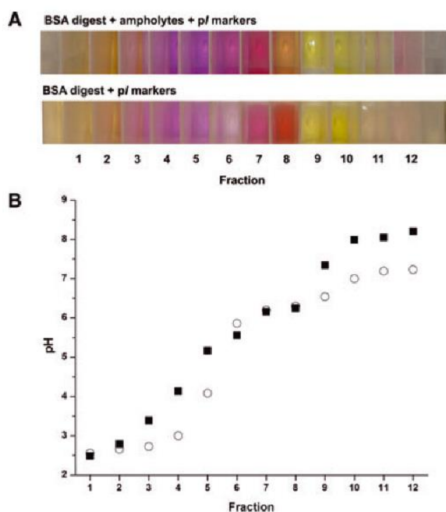

Figure 2. Collected fractions after IEF separation of BSA digest with the color pI markers in the presence and the absence of carrier ampholytes (A) and the plot of the pH values measured in the collected fractions (○, IEF in the absence of carrier ampholytes; ■, IEF in the presence of carrier ampholytes).

collected by the DF IEF device. The UV traces of chromatograms clearly demonstrate the differences in representation of peptide fragments in individual fractions with the most intensive peptide peaks presented in the acidic fractions. However, the chromatograms of the fraction 1 and original peptide mixture are almost identical. This is caused by a slow migration of the peptides in the high conductivity zones formed in the marginal regions of the DF IEF device. In order to compare the efficiency of the developed DF IEF

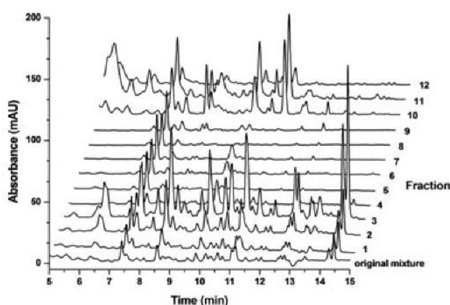

Figure 3. RP-HPLC separation of peptides fractionated using the DF IEF device. HPLC conditions: linear gradient, 5–35% solvent B in 12 min; injection volume, 7.5 μL; detection, 214 nm.

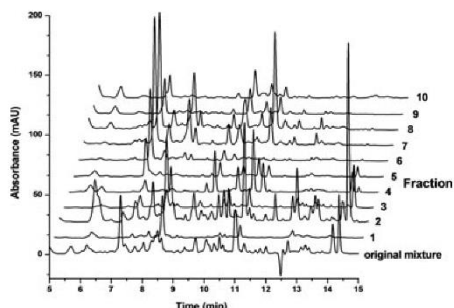

Figure 4. RP-HPLC separation of peptides fractionated using the MicroRotofor multicompartment electrolyzer. HPLC conditions: linear gradient, 5–35% solvent B in 12 min; injection volume, 7.5 μL; detection, 214 nm.

device with a commercially available instrumentation, the peptide mixture was also separated using the MicroRotofor multicompartment electrolyzer working in the autofocusing mode. Separation of the peptide mixture resulted in ten collected fractions that were subsequently analyzed using RP-HPLC. Corresponding chromatograms are shown in Fig. 4. In comparison to DF IEF, the peptides separated using the MicroRotofor device cover all the individual fractions. However, no final conclusions based only on the chromatographic separation with UV detection can be made.

Therefore, the collected fractions were also analyzed by ESI/TOF-MS. The use of the autofocusing mode was more suitable for direct MS analysis since carrier ampholytes could cause undesirable ionization suppression as demonstrated earlier [23]. Although some peptides were well focused and identified only in one fraction; some of them were spread across several fractions in similar concentrations. It might be caused by their very small net charge or electrically neutral nature over a broad pH range. This is especially the case of peptides having charge only on the C- and N-termini.

The peptide fragments were identified using the Protein Prospector database search using MS signals in the range of 400-1500 Da with signal intensity higher than five-fold of the baseline noise. The comparison of the sequence coverage obtained without the sample preseparation, and with fractionation using DF IEF or MicroRotofor devices is summarized in Table 1. Compared to a direct infusion of the original mixture, autofocusing using the DF IEF device provided significantly higher sequence coverage of all proteins used in this

Table 1. Comparison of the sequence coverage of proteins

	BSA	Cytochrome c	Myoglobin
Original mixture	22.1% (129/583)	27.9% (29/104)	35.9% (55/153)
DF IEF device	59.2% (345/583)	56.7% (59/104)	53.6% (82/153)
MicroRotofor	56.8% (331/583)	60.6% (63/104)	39.9% (61/153)

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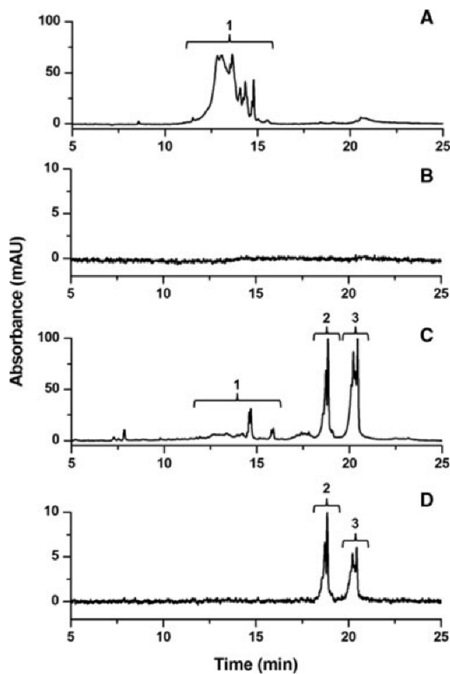

Figure 5. RP-HPLC separation of CMP standard (A, B) and unfractionated whey (C, D). HPLC conditions: linear gradient, 5–80% solvent B in 30 min; injection volume, 4 μL; detection, 214 nm (A, C) and 280 nm (B, D). Peaks: caseinomacropeptide (1), α-lactalbumin (2), β-lactoglobulin (3).

study. In addition to the separation process, desalting and concentrating effects of DF IEF improved the subsequent MS analysis and protein identification. Furthermore, the DF IEF device provided the preseparation efficiency comparable to the commercially available MicroRotofor device as demonstrated by protein sequence coverage in Table 1.

3.3 Isolation of CMP from whey

Another objective of this study was to evaluate the utility of preparative analysis/isolation based on IEF by the developed DF IEF device. Caseinomacropeptide (CMP) known also as glycomacropeptide from bovine whey was chosen as a model sample. Next to α-lactalbumin and β-lactoglobulin, CMP is the most abundant peptide/protein in whey and represents a heterogeneous group of phosphorylated and glycosylated polypeptides. Up to 11 genetic variants of a precursor of CMP, κ-casein, are known, but two genetic variants of CMP (A and B) dominate in bovine milk [38]. CMP is composed of 64 amino acid residues with molecular weight of 7 kDa; however, oligomerization of CMP under acidic and neutral pH conditions increases the molecular weight up to 50 kDa. CMP can trigger various physiological effects in a human body [38, 39]. Since CMP lacks aromatic amino acids including phenylalanine in its sequence, CMP is very often used as ingredients in diets designed for people suffering from phenylketonuria [40]. For this reason, improved processes are still needed in order to increase the purity of CMP and reduce phenylalanine content. Isoelectric point of CMP depends on the type of glycosylation and varies from 3.15 to 4.15 [41], whereas p*I* values of other major proteins of whey are above 4.3. Thus, DF IEF could be used as an efficient tool for isolation of CMP.

As mentioned above, CMP represents a heterogeneous mixture of peptides with no aromatic amino acids in the sequence. This fact was proved by RP-HPLC analysis of a CMP standard and whey with UV detection at 214 nm and 280 nm. As shown in Fig. 5, no significant amount of CMP standard was detected at 280 nm (Fig. 5B), although a series of peaks in the range from 12.0 to 16.0 min corresponding to CMP was detected at 214 nm (Fig. 5A). Similar results were obtained in the separation of whey samples, where only peaks corresponding to α-lactalbumin (19.0 min) and β-lactoglobulin (20.5 min) were detected at 280 nm (Fig. 5D).

In order to accommodate a larger volume of the sample containing high concentrations of proteins, peptides, and salts, a DF IEF device with a longer separation/isolation bed and three pairs of electrodes (Fig. 1B) was used for CMP isolation [31]. The simple preparative procedure included only dissolving whey powder and introducing the sample to the separation/isolation bed of the DF IEF device. After separation, the collected fractions were analyzed by RP-HPLC with UV detection. Chromatograms of untreated whey and separated fractions are shown in Fig. 6. The peaks of α-lactalbumin and β-lactoglobulin dominate the chromatograms obtained at 214 nm. After DF IEF analysis, α-lactalbumin was collected in fractions 6 and 7, and β-lactoglobulin in fractions 7 and 8. Based on the linear calibration curves ($R^2 = 0.999$) for α-lactalbumin and β-lactoglobulin content, the concentration of these two major contaminants decreased 3 times and 11 times, respectively, after DF IEF in comparison to the original sample of whey. The significant losses of these two proteins were mainly caused by their precipitation at the beginning of the separation/isolation bed of the DF IEF device. Due to the very low p*I*s, CMP peptides were detected in fractions 1–5 without a presence of α-lactalbumin and β-lactoglobulin as shown in Fig. 6 (18.0–21.0 min). Using the detection at 280 nm (data not shown), we confirmed that there was not any contamination of these fractions by other peptides/proteins containing phenyl groups. The series of peaks of CMP was detected between 12.0 min and 16.0 min with two major peaks corresponding to nonglycosylated variants A and B at 14.6 min and 15.8 min. Due to the high heterogeneity of CMP, the efficiency of CMP isolation and concentration of CMP in individual fractions were expressed in the percentage increase in comparison to the CMP amount in the original whey sample (Fig. 7). Almost 6-fold and 5-fold increase in CMP

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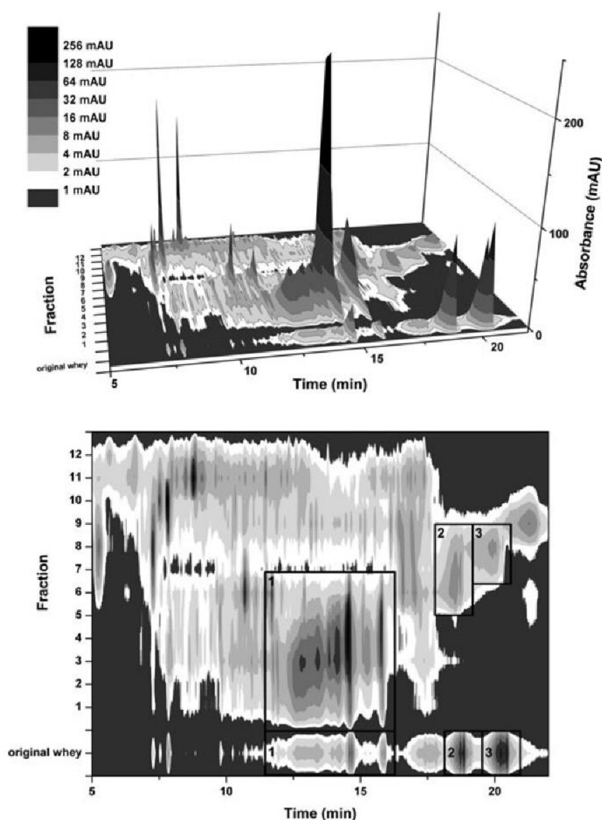

Figure 6. RP-HPLC separation of whey fractionated using the DF IEF device. HPLC conditions: linear gradient, 5–80% solvent B in 30 min; injection volume, 4 μL; detection, 214 nm. Peaks: caseinomacropeptide (1), α-lactalbumin (2), β-lactoglobulin (3).

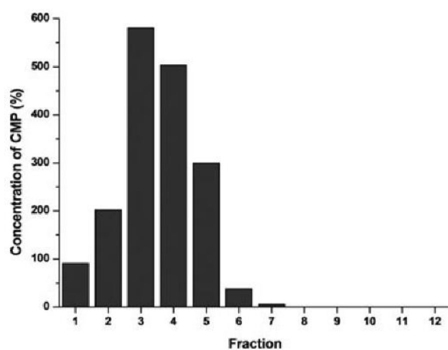

Figure 7. Concentration of CMP in individual DF IEF fractions.

concentration compared to the unfractionated sample was found in fractions 3 and 4. These results clearly demonstrate the effective isolation of CMP with significant concentrating effect using the DF IEF device.

4 Concluding remarks

The developed DF IEF device operating in the autofocusing mode was used for fractionation of peptide mixtures. We have verified that peptides obtained from complex tryptic digests can form the pH gradient and autofocus during the DF IEF analysis thus eliminating high-carrier ampholyte consumption. Furthermore, the use of the autofocusing mode eliminated the problems associated with the presence of carrier ampholytes in collected fractions subjected to subsequent MS analysis. Preseparation of the peptide mixture using DF IEF significantly improved protein sequence coverage. As

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demonstrated by the isolation of CMP from a crude whey solution, the DF IEF device might also serve as an efficient separation tool for preparative analysis. In addition, no carrier ampholytes contaminate the collected fractions, which make the method an attractive tool for direct analysis of the isolated peptides/proteins in many fields, for example, in biotechnology and food processing industry.

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The authors have declared no conflict of interest.

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3.2 Research article II

New solution IEF device for micropreparative separation of peptides and proteins

Possible drawback of free-flow IEF approach is necessary application of reasonable sample volume which means at least units or tens of milliliters. On the contrary, when solution IEF is employed, tens or hundreds of microliters suffice depending on a separation bed size. Hence, this was one of motivations leading to the invention and to the following patent of the solution IEF device based on nonwoven fabric by Šlais [176]. The solution IEF device was designed for micropreparative analysis of sample volumes in the range from 100 µl to several milliliters if larger focusing trays were used.

In the following paper, the above mentioned device was employed for optimization of IEF separation of colored p*I* markers and later, proteins and peptides contained in bovine sweet whey were analyzed using the device. Nonwoven fabric offers a few potential benefits regarding its characteristics. Since whole IEF proceeds in a separation bed made from strip of nonwoven fabric, there is no loss of proteins due to transport through a gel matrix (e.g. OFFGEL, ZOOM IEF Fractionator) or membranes (Rotofor) although some proteins can be lost due to adsorption on fibers of the fabric. Whole device is made from affordable parts and can be easily made in lab and in higher number for parallel runs. Nonwoven fabric strips have almost negligible cost compared to commercially produced IPG strips used for similar applications and furthermore their disposable character prevents cross-contamination between particular runs. Moreover, an easy protocol for complete fraction extraction can be applied using only disposable pipette tips.

Whey fractionation on the micropreparative solution IEF device was completed after approximately 12 hours, which determines it as an overnight technique and running time is comparable to already established IPG-based devices although Rotofor can fractionate in 3-4 hours. Furthermore, modification of original simple CA and buffers mixture by addition of 1-imidazoleacetic acid enabled better separation of CMP. Fractionation quality was subsequently tested by analysis of fractions in the second dimension – capillary RPLC. Two-dimensional results were processed into 3D separation map employing UV-DAD absorbance as the third dimension. The separation showed good separation of CMP cluster from α-Lactalbumin and β-Lactoglobulin and this time no precipitation was observed, which could be due to usage of high concentrations of ethylene glycol and glycerol.

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Research Article

New solution IEF device for micropreparative separation of peptides and proteins

The article presents a new concept of preparative solution IEF where time requirements and efficiency are similar to gel-based IEF whereas simple fraction handling as well as quick and complete protein recovery typical for solution-based IEF methods are maintained. The presented method is based on the IEF in separation medium soaked in a segmented strip of nonwoven fabric. The strip is positioned in an open horizontal V-shaped trough. Suggested focusing method combines free solution IEF under continuous evaporation and whole channel dispensing. Separation medium based on ethylene glycol/water mixture enhances viscosity enough to reduce electroosmosis and prevents the medium from completely drying out. Generation of pH gradient and final local pH is visually traced by colored low-molecular p*I* markers added to input mixture, which enables an optimization of focusing process and collection of individual fractions at desired pH range. The proposed method was tested by fractionation of the proteins and bioactive peptides originating from raw whey. Moreover, subsequent HPLC analysis of the individually collected solution IEF fractions was used for identification of whey components. We confirmed that the method is capable to process directly few tenths of milliliters of raw samples including the salty ones.

Keywords:
Bioactive peptides / Caseinomacropeptide / Micropreparative / Solution isoelectric focusing / Whey DOI 10.1002/elps.201200485

1 Introduction

Sample preparation is one of the most crucial processes in research of proteins and peptides. Raw protein mixtures obtained from various biological matrices are usually too complex. Thus, sample pretreatment connected with removal of salts and other low-molecular-mass substances is required prior to the analysis as well as a sample analyte preconcentration.

IEF is one of the methods used for this purpose and it is a particularly effective means of separation of complex proteins and peptides mixtures [1, 2]. IEF enables to concentrate analyte from a bulky sample solution; moreover, IEF provides knowledge about a protein p*I*, which can help with its identification. Although IEF is better known in gel format, solution IEF (sIEF) has also become popular [1–5].

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Abbreviations: CMP, caseinomacropeptide; **EG**, ethylene glycol; **IMAC**, 1-imidazoleacetic acid; **PVC**, polyvinyl chloride; **sIEF**, solution isoelectric focusing; **α-LA**, α-lactalbumin; **β-LG**, β-lactoglobulin

sIEF devices vary greatly in volume capacity as well as in resolution. Further, currently available preparative sIEF apparatuses solve disturbing effect of electroosmosis by various anticonvective strategies. These strategies include dividing a separation solution into the multicompartment channel where the compartments are separated by semipermeable membranes [6–11], mixing the separation medium with Sephadex beads [12], soaking the separation medium into a polymeric sponge [13], contacting the separation medium in isolated compartments by gel with IPG (off-gel IEF) [14, 15], contacting the separation medium soaked in isolated paper strips by gel with IPG [16], placing the separation medium into the series of parallel holes closed by gel with immobilized pH (parallel chip IEF) [17], filling an open separation channel with array of micropillars [18], and using a multiple-junction capillary fractionator [19]. Moreover, open surface of liquid sometimes causes water evaporation and this problem is hindered by extra cover or addition of liquid [15]. Finally, the salty protein samples often cause complications in separation by IEF [20]. Despite the number of examined concepts mentioned above, considerable improvement of sIEF can be seen permanently. Alternatively, fractionation of the sample can be done continuously by the free-flow IEF instruments [5, 21] or by divergent flow IEF [22–24].

Colour Online: See the article online to view Figs. 1–5 in colour.

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In our new concept of sIEF, focusing takes place in a separation medium soaked into a strip (preferably segmented) of a nonwoven fabric, which is inserted into an open horizontal V-shaped trough. The nonwoven fabric was recently used as an anticonvective bed for the continuous flow IEF [22–24]. Further, usage of an ethylene glycol (EG)/water-based separation medium enhances protein solubility, increases viscosity sufficiently to reduce electroosmosis, and prevents the medium from completely drying out. Suggested focusing method combines free-solution IEF under continuous evaporation with a whole-channel sampling. The pH gradient is generated by a series of several spacers added to the sample solution [25–28]. Moreover, the number of the spacers can vary and optionally it can be decreased so that the focusing approaches the mode of isoelectric trapping [9, 29]. All used spacers are defined, commercially available, and low-molecular-mass buffers. They are compatible with downstream analytical methods including LC with UV detection. Besides, a profile of pH gradient can be visually monitored by colored low molecular p*I* markers [28, 30–32]. It should be stressed that knowledge of pH gradient profile facilitates optimization of the focusing process and fraction collection after focusing.

Expected benefits of suggested method should be easy fraction handling, quick and complete protein recovery along with fair separation efficiency. Additionally, high concentration ratio of analyte in an obtained protein zone to the one in an original sample solution is also anticipated due to combination of focusing effect and concentration by water evaporation. Eventually, the method enables to process submilliliter volumes of raw tissue extracts including salty ones.

Feasibility of the new method was tested by fractionation of a solution containing colored p*I* markers and proteins/peptides originating from whey. Whey as a major cheese making byproduct contains many valuable bioactive substances. One of the interesting major whey components is caseinomacropeptide (CMP). It is a 64 amino acids long polypeptide originating by a cleavage of κ-casein by chymosin [33]. However, experimental molar mass is a complex issue [34]. CMP belongs to the group of whey bioactive peptides with various beneficiary health effects [35]. In the structure of CMP, there are no aromatic amino acids and thus CMP is suitable for a diet of people with hepatic diseases [36]. Recently, we have published a paper based on divergent flow IEF separation of major whey proteins/peptides [37].

2 Materials and methods

2.1 Materials chemical instruments

Dried whey powder was obtained from ASP CZECH, Slušovice, Czech Republic. α-lactalbumin (α-LA), β-lactoglobulin (β-LG), CMP, glutamic acid, ACN, butan-1-ol, HEPES, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPS), glycyl-glycine (DIGLY), patent blue V sodium salt, creatinine, histidine, 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol, imidazole, Tris, THF, TFA, ammediol, *N*-methyl-glucamine, EG, and sucrose were obtained from Sigma-Aldrich (St. Louis, MO). 1-Imidazoleacetic acid (IMAC) was purchased from TCI Europe, Zwijndrecht, Belgium. Dissociation constants of IMAC acid $pK_1 = 2.8$, $pK_2 = 6.8$, and $pI = 4.8$ were determined by potentiometric titration at 25°C. Colored p*I* markers were developed and prepared in authors' laboratory mainly by diazotation and/or Mannich reaction [27, 28, 30–32]. Distilled water was prepared fresh in laboratory.

Consort programmable laboratory power supply EV232 with the maximum voltage of 3000 V (Consort bvba, Turnhout, Belgium) was used for all IEF separations. Portamess 913 pH meter (Knick, Berlin, Germany) with Biotrode μ-pH electrode (Hamilton Bonaduz, Bonaduz, Switzerland) was used for measuring pH in fractions.

Table 1. Composition of stock solution of simple buffers

Substance	pK1	pK2	Concentration in stock (mmol/L)
Iminodiacetic acid	1.8	2.6	2
Aspartic acid	1.9	3.7	2
Glutamic acid	2.2	4.3	5
1-Imidazoleacetic acid	2.8	6.8	2
HEPES	3.1	7.5	2
EPPS	3.8	7.9	15
DIGLY	3.2	8.2	15
Creatinine	4.8	9.2	10
Histidine	6.0	9.3	10
BISTRIS	6.5	–	15
Imidazole	7.1	–	15
Tris	8.3	–	2
Ammediol	8.8	–	2

EPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; DIGLY, glycyl-glycine.

2.2 Input solutions

In all analyses, total volume of 0.7 mL was used for sIEF. Testing mixture of defined buffers and p*I* markers consisted of 0.15 mL of stock solution of 12 spacers (see Table 1) and sodium hydroxide, 0.05 mL of stock solution of colored p*I* markers (for description of structures of markers and their colors, see Table 2), 0.15 mL EG, 0.05 mL butan-1-ol, and 0.3 mL of water. IEF whey sample mixture was composed of 0.375 mL of 1% w/v solution of dried whey, 0.05 mL of stock solution of colored p*I* markers (Table 2), 0.1 mL EG, 0.05 mL butan-1-ol, 0.025 mL 0.1 mol/L IMAC, and 0.1 mL of 0.1 mol/L Tris.

2.3 Micropreparative sIEF device

Separation took place in a liquid medium soaked in a strip made from nonwoven fabric located in a V-shaped trough (see Figs. 1 and 2). The trough was made of a white 0.25 mm

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Table 2. Colored markers used for sIEF experiments

Number	Name	p*I*	Concentration in stock g/L	Color
I	Patent Blue V	2.0	0.1	Green
II	2-({4-[Bis(2-hydroxyethyl)amino]phenyl}azo)terephthalic acid	2.8	0.2	Red
III	5-(Dimethylamino)-2-[(3-pyridyl)azo]benzoic acid	3.9	0.2	Orange
IV	3′,3′′-Bis(4-morpholinomethyl)-5′,5′′-dichlorophenol-sulfonephthalein	5.3	0.2	Lavender
V	2-{[3,5-Bis(4-morpholinomethyl)-4-hydroxyphenyl]azo}benzoic acid	5.7	0.4	Yellow
VI	3′,3′′-Bis(4-morpholinomethyl)-o-cresolsulfonphthalein	6.2	0.4	Red
VII	4-Methyl-6-nitro-2-(4-morpholinomethyl)phenol	7.2	0.4	Yellow
VIII	2,6-Bis[4-(morpholinomethyl)]-4-[2-(thiazoyl)azo]phenol	8.0	0.2	Orange
IX	4-Methyl-6-nitro-2-[(3-dimethylamino)propylaminomethyl]phenol	9.0	0.4	Yellow
X	3′,5′,3′′-Tri(*N,N* dimethylaminomethyl)-phenolphthalein	10.0	0.5	Violet
XI	3′,5′,3′′,5′′-Tetrakis(*N,N* dimethylaminomethyl)-phenolphthalein	11.0	0.5	Lavender

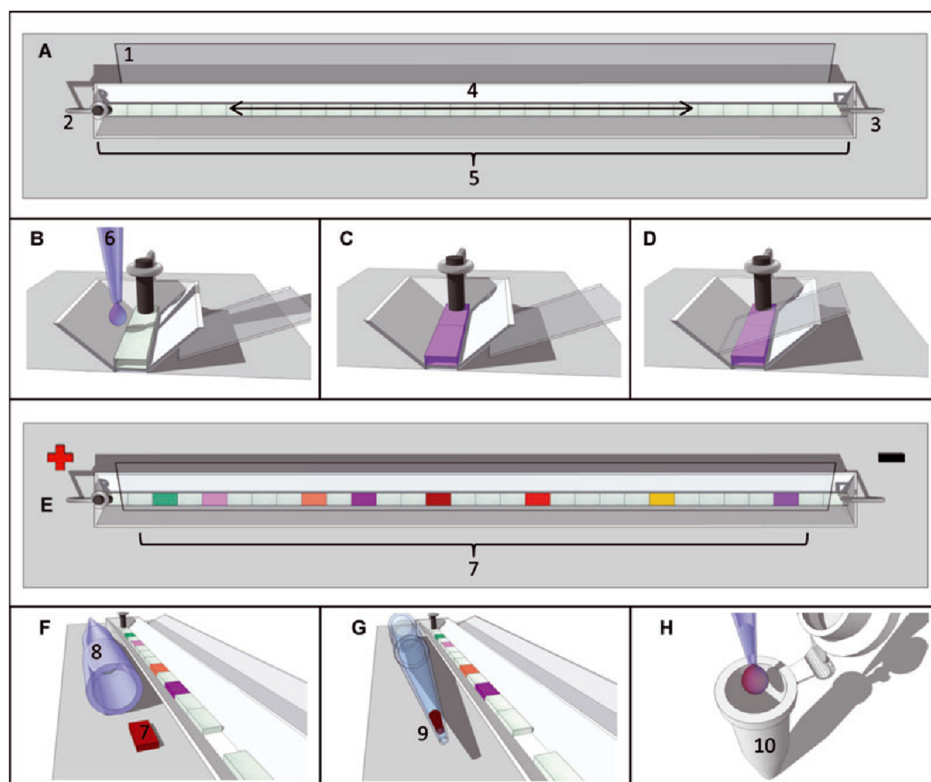

Figure 1. sIEF device operating protocol. (A) Image of the whole device: (1) clear plastic cover foil, (2) carbon anode, (3) stainless wire cathode, (4) segmented nonwoven fabric strip, (5) plastic trough; (B) dispensing of an input mixture ((6) pipette tip); (C) the input mixture soaked in the strip; (D) a cover foil is placed over the strip; (E) result of IEF run ((7) separated colored p*I* markers); (F) one segment of the strip—IEF fraction—picked up from the trough ((8) clean pipette tip for fraction extraction); (G) fraction pressed in the top of pipette tip ((9) fraction); (H) elution of fraction into micro test tube (10).

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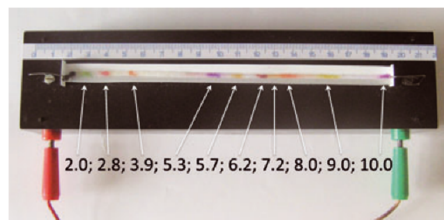

Figure 2. Device after focusing run with simple buffers and colored p*I* markers. p*I*s of particular markers are labeled by arrows.

thick polyvinyl chloride (PVC) sheet Durofol (FATRA, Napajedla, Czech Republic). The PVC sheet was cut and bent on a guillotine paper cutter and subsequently individual parts were glued together by THF. Length of the trough was 175 mm. Furthermore, part of an anode was made of 2 mm graphite rod while an anode holder and a cathode were made of the Osteofix 0.6 mm stainless steel wire (Železárny Chomutov, Chomutov, Czech Republic). The whole set-up was then mounted on the top of a molded plastic box with electrode connection wires hidden underneath.

Concerning the separation bed, segments of a nonwoven fabric of 6 mm in length, 2 mm in width, and 0.5 mm in height were cut from a 0.5 mm sheet of the polyester Vliseline-type nonwoven fabric Novoline, 80 g.m^{-2} (Polytex, Malé Svatoňovice, Czech Republic). The sheet was cut by the guillotine paper cutter. The segments were then aligned inside the trough and glued by drying out 5% sucrose solution. Total length of the separation bed composed from series of these segments was derived from the length of the trough and reached 175 mm. Alternatively, the sIEF device can be made of a nonsegmented strip of the nonwoven fabric 175 × 2 × 0.5 mm located in the trough described above.

2.4 sIEF method

The overview of the sIEF fractionation protocol is illustrated in Fig. 1. Generally, the separation bed is made of a single piece of strip or preferably assembled from the precut segments of a strip of a nonwoven fabric located in the trough, see Fig. 1A. At the beginning, input solution is dispensed by a pipette into the horizontally positioned trough with the aligned nonwoven segments (Fig. 1). The solution then soaks into the fabric uniformly over the trough length (Fig. 1C). After sampling is done, the trough is covered by a transparent PVC foil of 20 × 165 mm (Fig. 1D) and electrodes are connected to programmable power supply. Subsequently, the whole device is inserted into a cooling box under the temperature of 10°C and 50–60% of relative humidity. The power supply is switched on, and then focusing proceeds overnight. Voltage is programmed to increase from 100 to 200 V over 4 h, then voltage is increased from 200 to 1000 V over 4 h, and finally, voltage is increased from 1000 to 3000 V over 4 h. When voltage reaches 3000 V, this value is maintained until the fractions are collected. Most of the water from sample solution evaporates throughout the focusing period. The inspection of the IEF run can be made conveniently with the use of colored ampholytes [23, 24, 27, 28, 32, 38], see also [10, 14]. Advantageously, we used here our synthetic low-molecular-mass p*I* markers.

After overnight focusing the device is taken out of the cooling box, the cover foil is removed, and a result of the focusing is indicated by positions of the colored p*I* markers, see Fig. 1E. Individual segments are then manually picked up by tweezers (Fig. 1F) and fixed into 1 mL pipette tips by pressing with the free end of a plastic single use inoculation loop, see Fig. 1G. Finally, content of each segment (fraction) is washed out with 100 μL of water into micro test tubes, see Fig. 1H.

2.5 RPLC

Liquid fractions obtained from sIEF were analyzed by the micro RPLC. All chromatographic separations were carried out at the Agilent 1200 Series chromatographic system (Agilent Technologies, Santa Clara, CA, USA). Microbore Poroshell 300SB-C18 column (5 μm particle size, 1 × 75 mm, Agilent Technologies) equipped with the C18 cartridge guard was used for all separations. Elution was run at the flow rate of 20 μL/min and 70°C by the binary gradient of water with ACN as an organic modifier. Solvent A consisted of 0.1% v/v TFA in water and solvent B consisted of 0.1% v/v TFA in ACN. Linear gradient from 5 to 80% v/v of solvent B over 30 min was used for all separations. Sample injection volume was set to 4 μL. Gradient delay of the system was 5 min. Detection was performed at 214 and 280 nm using the DAD (Agilent 1200 G1315D DAD detector). In addition to sIEF whey fractions, 1 g/L solutions of standard whey proteins were also analyzed.

3 Results and discussion

3.1 Device testing

In the beginning, various profiles and sizes of separation vessel along with different thickness and width of a strip were tested. Furthermore, effect of several additives (including surface tension modifiers and substances altering viscosity) was also tested. Presented basis of input mixture resulted with the best separation efficiency.

At preliminary separations, the device with a nonsegmented strip was operated using a mixture of simple buffers (Table 1) and colored p*I* markers (Table 2). The mixture was designed to demonstrate separated color zones of the p*I* markers after a complete IEF run. This feature enabled easy observation of focusing progress. Moreover, this visual monitoring enabled us quick recognition of the issues throughout the focusing experiment. Besides, any considerable Joule heating would be easily recognized by water condensation on the cover foil but none was observed during the experiments. Temperature of 10°C and relative humidity of 50–60% led

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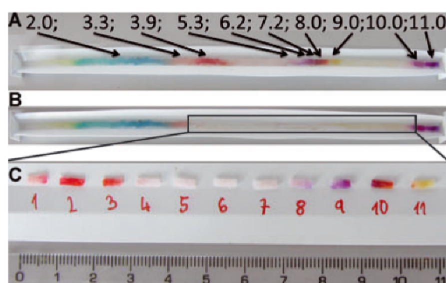

Figure 3. Device after focusing run with whey solution and extraction of obtained fractions. (A) Image of the trough after separation of whey mixture, particular p*I*s of markers are labeled by arrows. (B) Image of the trough with segments of a strip within pH range 3–9 removed. (C) Detailed image of segments of a strip (sIEF fractions) within pH range 3–9 positioned separately and numbered.

water to evaporate during sIEF run and EG in the fractions reached concentration equilibrium about 80% v/v (from 15% v/v starting concentration), as was estimated from vapor pressure diagram of aqueous EG. Evaporation increased viscosity of the input solution close to 15 cP and reduced EOF sufficiently to keep proteins focused.

Further, butan-1-ol was used for surface tension modification of the nonwoven fabric surface. Usage of this additive facilitated liquid soaking into a strip. Butan-1-ol evaporated during focusing run and eventual additive residue was neglected in further processing of collected fractions by RPLC.

An image of the whole device and a detailed image of the strip with separated colored markers were taken after focusing the mixture of simple buffers with the p*I* markers (see Fig. 2 and also a device operating protocol—Fig. 1E). Figure 2 shows p*I*s of the particular markers with arrows pointing to the corresponding colored zones. Even though simple background ampholytes were used, all ten p*I* markers were separated in distinct zones.

Demonstrated IEF separation device proved good day-to-day repeatability of positions of colored zones (data not shown). Moreover, the suggested method can be easily optimized by adjusting voltage/current characteristics to reduce overheating.

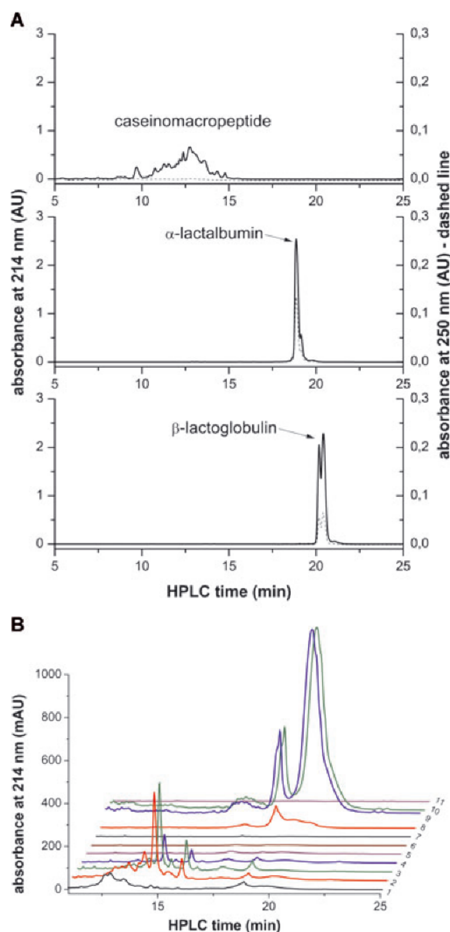

Figure 4. RPLC chromatograms of protein standards and sIEF fractions. (A) LC chromatograms of the CMP, α-LA, and β-LG; conditions of the separation are described in the experimental part (see Section 2.4). (B) LC profiles of the IEF fractions (numbered from the anodic side); separation conditions are described in experimental part, see Section 2.4.

3.2 Separation of whey proteins

A solution prepared from dissolved dried whey was focused according to the protocol described in part 2.3, which was optimized for the separation of CMP from whey proteins (major contaminants α-LA and β-LG with masses of 14.2 and 18.3 kDa, respectively [39]). Result of the sIEF separation is shown in Fig. 3A (see also Fig. 1E) and collected fractions are shown in Fig. 3B and C (see also Fig. 1F). It was found by differential weighting that final weight of one fraction (sizes of segment defined earlier) was about 10 mg, which is approximately 10 μL.

Quality of the focusing was examined by HPLC analysis of sIEF fractions. HPLC analysis of the IEF fractions was a final step in identification and a quantification of peptides/proteins originated in the whey sample. Analysis of standards of CMP, α-LA, and β-LG (see Fig. 4) led us to identify them in chromatograms of the fractions. With regard to

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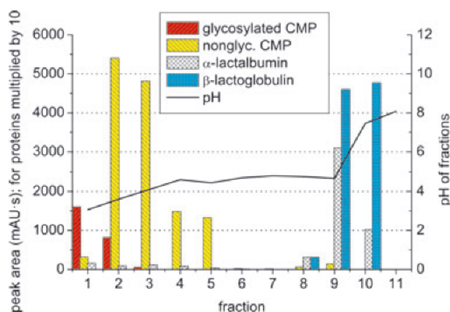

Figure 5. Bar graph—concentration of proteins/CMP in sIEF fractions and corresponding pH. The peak areas (detection at 214 nm) of individual peptides/proteins from whey prefractionated with the micropreparative solution IEF device, line represents pH in fractions (values on the right axis).

supposed p*I*s of CMP, α-LA, and β-LG, sIEF fractions from region of pH 3–9 were chosen for RPLC analysis. After analysis of chromatograms for individual fractions by CHEMSTATION software (Agilent Technologies), the integrated peak areas of glycosylated CMP (12th–15th min), nonglycosylated CMP (15th–17th min), α-LA (19th min), and β-LG (21st min) were processed into the colored bar graph (see Fig. 5). Scales for α-LA and β-LG were ten times higher than for CMP. Furthermore, pH corresponding to each fraction was measured with the pH electrode and it was marked in the same graph as full line. With respect to presented graph, only low percentage of α-LA was found in fractions occupied with CMP and no β-LG was detected in these fractions.

Moreover, measured pH of the collected fractions was compared with p*I* of p*I* markers (Fig. 5). Components in the whey sample formed a nonlinear pH gradient along the strip during the focusing process (see Fig. 5—pH is shown as a line with an appropriate axis on the right). Hence, no more additional ampholytes were necessary. CMP as a heterogeneous group of diversely glycosylated peptides varies its p*I* within the broad range from 3.15 to 4.15 [40]. Glycosylated CMPs have p*I*s lower than nonglycosylated variants due to presence of the frequent saccharide in CMP glycans—*N*-acetylneuraminic acid pKa = 2.2 [40,41]. Graph of concentration of CMP in the fractions after IEF run (Fig. 5) clearly demonstrate that glycosylated part of CMP has more acidic p*I*. The p*I*s of α-LA and β-LG are more basic, that is, 4.2–4.5 and 5.13, respectively [39]. Therefore, IMAC with p*I* = 4.8 was used as p*I* spacer in order to get better separation of CMP from other whey proteins. Our results showed that p*I* of IMAC is in the interval of CMP p*I* and p*I*s of two major whey proteins α-LA and β-LG, although literature presents p*I* of α-LA lower than IMAC p*I* [39].

Finally, protein recovery was calculated from the sum of LC peak areas of the particular substance (UV detection at 214 nm) in all collected fractions, which was compared to the particular LC peak area of the substance in the input whey solution. In detail, it means that a total amount of each protein (100%) in one sIEF run was determined from the LC chromatogram of 2% whey w/v, then this value was divided by its dilution factor in the input mixture (3.2) and multiplied by 30 (it was assumed that this amount is in each of the 30 segments at the beginning of sIEF run). Recovery of glycosylated and nonglycosylated CMP was calculated to be 44 and 80%, respectively. Recovery of α-LA and β-LG was found out to be 77 and 101%, respectively. Low recovery of the glycosylated CMP could be caused by its slow migration from the acidic region, which was not collected. Presented values are only preliminary, due to the approximation of the volume of each segment that impacts ongoing dilution during segments washing.

4 Concluding remarks

We present a new device for sIEF in a liquid medium soaked into segmented nonwoven fabric strip. Usage of commercially available and defined simple buffers enabled the method to be compatible with downstream LC analysis with UV detection. Moreover, the simple buffers contributed to overall reproducibility and economy of the analysis. The pH gradient profile and the local pH were visually traced by colored low molecular p*I* markers. This feature enabled the optimization of the focusing process and subsequent fraction collection despite the location change of fractions due to various salt and analyte concentrations in the samples.

One of the significant benefits of suggested method is its concentrating effect. Concentration of analytes in focused zone was increased more than one order of magnitude and it was reached not only by IEF, but also by water evaporation. To simplify the fraction collection, a few-step protocol with minimum manipulation steps was employed for extraction of microliter volume fractions from strip segments.

In order to demonstrate capability to separate raw biological samples, dissolved dried whey representing raw protein mixture was fractionated on the novel sIEF device. Subsequent HPLC analysis confirmed the efficiency of the whey sIEF separation. CMP was separated from two major contaminating proteins α-LA and β-LG. On basis of these results, the presented method has proved to be an efficient tool for the purification of proteins/peptides from raw complex protein mixtures.

It is worth mentioning that several features of the developed device are superior to current devices for sIEF. First, volume of fraction can be defined prior to an analysis using precut nonwoven fabric strip or after analysis by cutting an undivided strip. Moreover, size and position of segments is adjustable for different samples in regard to position of colored p*I* markers. Second, usage of pipette tips along with repetitive flushing is promising for providing an option of robotic manipulation and reproducible quantitative recovery of separated substances. At last but not least, the focusing vessel, strip, and all other items used for the fraction extraction

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are low cost and thus fully disposable. This feature eliminates sample cross-contamination. To conclude, we found out that the developed device is an efficient tool for proteins/peptides micropreparation.

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3.3 Research article III

Simple automated liquid chromatographic system for splitless nano column gradient separations

Two previous publications describe mainly the first dimension while capillary format liquid chromatograph for analysis in the second dimension is applied as an established method for analysis of proteins and peptides. Therefore, in the third paper effort was focused on development of splitless nano column liquid chromatographic system which could be employed as the second dimension of analysis considering samples of very small volumes and had possibility of online coupling.

High sensitivity and easy coupling to MS detectors using soft ionization techniques (matrix assisted laser desorption/ionization and eletrospray ionization) are the main reason why LC system working within nanoliter volume range are applied for analysis of peptides and proteins. Moreover, gradient mobile phase elution is almost exclusively applied for analysis of peptides and proteins. However, production of mobile phase gradient is not an easy task when nano columns (internal diameter 100 µm and less) are used. Up to now, manufacturers developed state of the art systems with a sophisticated hardware and software which were able to overcome all the limitations connected to transfer from analytical to nano scale LC. One of the used approaches is splitting of mobile phase gradient produced with common analytical scale pumps. Possible drawback is amount of unused mobile phase which is discarded without use. Hence, a splitless LC system able to create mobile phase gradient in nano scale could be advantageous in the field of two-dimensional separations employing mainly nano RPLC in the second dimension[67].

Main parts of newly devised nano LC system include programmable syringe pump with glass syringe, ten port selector valve, USB-device for system control, vials with mobile phase, separation column, and capillaries for connection. Main advantage of this device is genuine system of gradient creation using only one syringe pump which is controlled automatically. Assembled nano LC system was firstly tested by mobile phases where acetonitrile concentration was marked by uracil and gradient shape was optimized. Subsequently, the system was evaluated by successive runs of alkylphenones and peptides originating from tryptic digest of BSA. Results showed high repeatability between individual runs of alkylphenones and peptides separation; therefore, it was considered as suitable for nano RPLC analysis of proteomic samples.

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Simple automated liquid chromatographic system for splitless nano column gradient separations☆

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ABSTRACT

A simple splitless gradient liquid chromatographic system for micro and nano column separations has been assembled and tested. It consists of an OEM programmable syringe pump equipped with a glass microsyringe and ten-port selector valve. Gradient of mobile phase was created in the syringe barrel due to turbulent mixing. Capability of suggested system to create various gradient profiles was verified using 50-μl, 100-μl, and 250-μl glass syringes. Acetone, thiourea, and uracil were tested as gradient markers and finally, uracil was proved to be an excellent way of water–acetonitrile gradient tracing. It was found that up to 80% of the total syringe volume is available as a linear gradient section. In context to micro and nano column chromatography, the best results were obtained using the 100-μl syringe. Separations were performed on the capillary monolithic column Chromolith CapRod RP-18e (150 mm × 0.1 mm) and system performance was evaluated using a test mixture of six alkylphenones as well as tryptic digest of bovine serum albumin. Results proved that suggested system is able to create uniform gradients with high repeatability of retention times of test solutes (RSD < 0.3%). Repeatability of injection of sample volumes in the range of 0.1–3 μl was evaluated using on-column preconcentration technique which means that sample was diluted in mobile phase of low eluting strength. Repeatability of the peak areas was measured and statistically evaluated (RSD < 5%).

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1. Introduction

High performance liquid chromatography on micro and nano columns has become very relevant separation technique. Utilization of micro and nano columns is ideal for all sample-limited applications [1,2]. Other important benefit is flow rate compatibility of micro and nano columns with mass spectrometers and also better column temperature control. An application of gradient elution makes this separation technique extremely powerful for the separation of solutes of wide range of polarity, improves the solute detectability, and increases the speed of analysis [3,4]. These facts make micro and nano column liquid chromatography (LC) attractive for many researchers dealing with complex samples. However, employing micro and nano columns with standard HPLC gradient instrumentation is not effective due to splitting of major part of the mobile phase and large gradient delay volume. On the other hand, repeatable generation of splitless mobile phase gradients for these columns at the flow rates below 1 μl min^{-1} is not an easy task. Commercial systems have been placed on the market not long ago; nevertheless, they are bulky and expensive. Therefore, a simple and easily modifiable system may be adequate substitution of such complex and expensive instrumentation, and it may serve well in certain sectors (e.g., basic research, education). Several approaches to this problem have already been suggested. One of the first papers dealing with gradient micro-HPLC was published by Ishii et al. in the end of the 1970s [5]. They used an open tube for storing of a gradient prepared via exponential dilution method [6] before own separation. In more recent works [7–10], several designs of micro-exponential diluters based on actively mixed chamber have been described; nevertheless, convex shape of the gradients obtained in these systems could be disadvantageous in some applications. Therefore, other approaches have been investigated to offer different gradient profiles, especially, the linear one. Slais et al. described a mixing device consisting of two coaxial tubes [11]. While the outer tube serves as a mixer and initial solvent container, the inner one has a number of small holes along its length and the final solvent enters the mixer at several places resulting in increasing final solvent percentage in the liquid leaving the mixer. Employing the closing ring inside the mixer, selected holes could be closed

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and different gradient profiles could be performed including segmented, convex, and linear one. In another work [12], Slais et al. described an open-tubular mixer consisting of a coiled capillary and spiral shaped tube producing gradients of the sigmoidal profile and different steepness. Que et al. and Kahle et al. [13,14] utilized turbulences occurring in the place of sudden change of tubing diameter for mixing of two mobile phases to generate sigmoidal gradients with prolonged linear mid part for capillary electrochromatography. A couple of approaches employing more complex systems of one or more gradient loops have also been designed. Takeuchi et al. [15] built up a system for stepwise gradient elution consisting of two injection valves with two loops alternately filled and integrated into the flow. In more recent works, Davis et al. [16,17] used an injection valve and loop to store a linear gradient produced by the low pressure mixing of two solvents. Ito et al. and Deguchi et al. [18–20] used a ten port two-position valve with two loops to deliver the gradient produced by microflow pump to nano column at nanoflow rate. Natsume et al. [21] and Capiello et al. [22], they both developed systems consisting of a couple of gradient loops serving to store isocratic segments of the stepwise gradient produced by standard gradient instrumentation. A stepwise gradient at the corresponding flow rate is generated during the elution. A continuous gradient profile is obtained while the step edge is swept due to diffusion in the tubing [21] or after passing the mixing chamber [22]. Siviero et al. [23] have recently optimized and refined this configuration in the meaning of complete automation and simple operation. In the most recent works [24,25], a significant simplification of this approach has been achieved. Solvents are delivered here via isocratic micro- or nanoflow pumps and no additional valves are necessary. Murata et al. described a solution where the stepwise gradient is generated after merging of three flows created by splitting the final solvent flow into three tubes of different diameters initially filled up with the mobile phase of low eluting strength [24]. Step edges are consequently swept by passing through the mixing chamber. Brennen et al. utilized advantages of lab-on-chip technology to build up nanoflow LC chip [25]. Gradient generation section of the chip consists of twenty timing channels and stepwise gradient is prepared in the same manner as in Murata's system, but there is no need for mixing chamber since the small steps are relatively fast mixed due to diffusion.

Almost all of the devices and systems mentioned above suffer from some drawbacks. In the case of micro-exponential diluters it is the low flexibility in obtaining of various gradient profiles. The geometrical configuration of the mixers has to be altered to change the gradient profile. Moreover, the preparation of linear gradient profile is difficult. On the other hand, more flexible systems are usually too complex. Recently, we have published a simple laboratory set-up for proteomic purposes for peptide pre-concentration and separation by gradient elution [26] where gradient was prepared by successive sucking of four mobile phases with gradually decreasing acetonitrile concentration into inclined glass syringe. All procedures were carried out manually in this case. Following principles described previously [26,27] we developed an automated splitless gradient liquid chromatographic system for the gradient elution in micro and nano LC employing columns with the low backpressure. Capability of developed system to create linear gradient of various steepness, gradient repeatability as well as sample injection repeatability were studied in detail in this work.

2. Experimental part

2.1. Materials and chemicals

Alkylphenones were obtained from Aldrich (Milwaukee, WI). Test mixture was prepared by dissolution of alkylphenones (20 μM each) in acetonitrile–water (5:95, v/v). HPLC grade acetonitrile (ACN), uracil, bovine serum albumin, dithiothreitol (DTT), iodoacetic acid, and trifluoroacetic acid (TFA) were purchased from Sigma–Aldrich (Schnelldorf, Germany). Deionized water was prepared using a Milli-Q water purifier (Molsheim, France). Agilent DAD 1200 detector (G1315D, 80 nl detection cell with optical path length of 6 mm) was used to acquire UV-absorption spectra. The New Era NE-500 OEM syringe pump, ten-port selector valve C55-1340 I (Vici Valco, TX), and the Agilent VWD SL+ detector (G1314E, Agilent Technologies, Santa Clara, CA) equipped with a nano cell (SunChrom, Friedrichsdorf, Germany) with effective optical path of 0.17 mm were used for all other experiments. Separations were performed on capillary monolithic column Chromolith CapRod RP-18e 150 mm × 0.1 mm (Merck, Haar, Germany). The separation column was placed in LCO 102 column thermostat (Ecom, Prague, Czech Republic).

2.2. Gradient tracing

Standard way of gradient tracing in LC is the addition of acetone into one component of the mobile phase. Acetone is regularly used as a dead volume marker; however, bathochromic shift of its UV absorption maximum at 265 nm during the change of the mobile phase composition from pure water to acetonitrile is unfavorable. Moreover, the molar absorption coefficient of acetone is relatively low. Using a spectrophotometric detector with a nano cell, there is a relatively narrow range where the sensitivity is sufficient and the portion of acetone does not change the mobile phase composition substantially. Thiourea and uracil are another commonly used dead volume markers in the liquid chromatography. Molar absorption coefficients of these two compounds are about three orders of magnitude higher than the acetone's one. Absorption spectra of thiourea and uracil in solutions with gradually increasing acetonitrile concentrations were acquired in order to verify their suitability as the water–ACN gradient tracers. Measured solution was manually sucked into the glass syringe. The syringe was then connected to the Agilent DAD 1200 detector via short piece of a fused silica capillary of i.d. 50 μm. Adequate volume of the solution was delivered at the constant flow rate of 10 μl min^{-1} using the KDS-100-CE infusion syringe pump (kdScientific, Holliston, MA).

2.3. Splitless gradient chromatographic system

Experimental design of automated splitless gradient liquid chromatograph is schematically represented in Fig. 1. Suggested system consists of the NE-500 OEM programmable syringe pump (1) equipped with a glass microsyringe (2) (SGE Analytical Science, Victoria, Australia) and ten port selector valve C55-1340 I (3). It is essential that the syringe is in an inclined position under the angle of 20° with the needle pointing downwards. The heavier water-rich part of the mobile phase gradient is then located in the lower part of the syringe barrel. Syringe pump is controlled by sending commands in defined format via RS-232. The selector valve is controlled by switching two circuits – one for step forward and the second one to set the home position – port no. 1. Switching the circuits was performed by two 5 V relays (Finder, Almese, Italy) connected to digital outputs (0 V/5 V) of the NI USB-6009 data acquisition device (4) (National Instruments, Austin, TX). Whole system was controlled via simple software compiled in the LabVIEW development environment (National Instruments, Austin, TX). All hydraulic connections were performed using fused silica capillaries (MicroQuartz, Munich, Germany). Glass syringe was connected to the central port of the ten-port selection valve via short piece of the fused silica capillary (100 mm × 180 μm i.d., 360 μm, o.d.). This piece of capillary and also the syringe needle (55 mm × 250 μm, i.d.) served as an injection loop of volume about 5 μl (5). The first four

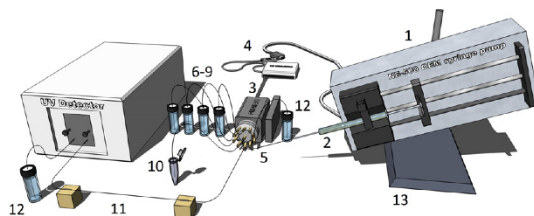

Fig. 1. Experimental design of developed system. (1) Programmable syringe pump, (2) glass syringe, (3) ten port selector valve, (4) NI-USB 6009 data acquisition device, (5) sample loop, (6–9) mobile phases, (10) sample, (11) separation column, (12) waste, (13) holder.

ports served as inlets for ACN–water mobile phases with gradually decreasing acetonitrile concentration (6–9). The fifth port served as the sample inlet (10) and the sixth one was used for connection of separation column (11). The seventh port served as a path to the waste (12) while remaining ports stayed unoccupied.

2.4. Instrument function

Before an analysis, suitable volume of the mobile phase of lowest eluting strength was sucked into the syringe and consequently pushed through the column to re-equilibrate it. Then the adequate volume of the final mobile phase was sucked into the syringe and discharged. This procedure was repeated five times to wash out the remaining low concentrated mobile phase from sample loop. After that the specific volumes of individual mobile phases with gradually decreasing acetonitrile concentration were successively sucked into the syringe. The S-shaped gradient of the mobile phase was created in the syringe barrel as a result of turbulent mixing [14] occuring at the syringe needle–barrel boundary (Fig. 2). The flow path was then switched and desirable volume of a sample from 0.1 μl to 3 μl was sucked into the sample loop. The analysis began after the flow path had switched to the column. On column focusation [28] proceeded due to low eluting strength of sample matrix.

In the presented configuration, the gradient delay is affected by a volume of sample introduced into the sample loop and mobile phase flow rate as well. This means that at low flow rates and low sample volumes the gradient delay could be unfavorable; e.g. using flow rate 0.5 μl min^{-1} and 0.1 μl of sample the gradient delay is approximately 10 min. However, the sample loop could be completely filled by a sample when on column focusing is involved. Thereafter the dwell volume as well as gradient delay would be minimized.

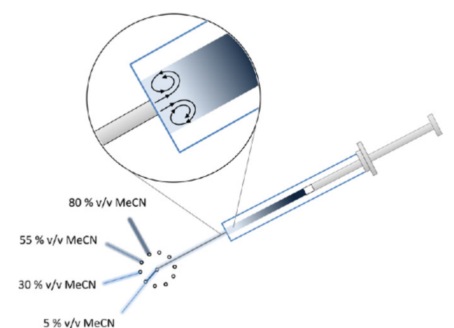

Fig. 2. Illustration of turbulent mixing at the point of abrupt change of the tubing diameter at syringe needle–barrel boundary.

Pressure resistance of the used glass syringe is up to 50 bars. From this point of view, the system may be seen as more appropriate for the monolithic columns. However, application of water-acetonitrile gradient at flow rates up to 0.5 μl min^{-1} on packed column of suitable parameters may also be considered, especially when elevated temperature will be applied.

2.5. In-solution digestion of BSA

Peptides for the analysis were prepared by a trypsin cleavage of bovine serum albumin (BSA) in the same way as in our previous publication [25]. Dithiothreitol and iodoacetic acid were used for the reduction of BSA disulphide bridges and following alkylation of the thiol groups. Then BSA was precipitated with trifluoroacetic acid at −20 °C. After centrifugation and discarding of the liquid, the pellets were dissolved in ammonium hydrogen carbonate buffer with trypsin in the 1:50 mass ratio to BSA. Digestion took part overnight and subsequently formed peptides were vacuum dried.

3. Results and discussion

3.1. Alternative gradient tracer

UV-absorption spectra of thiourea and uracil are shown in Fig. 3a and b, respectively. Unfortunately, thiourea showed significant shift of the absorption maximum throughout the change of acetonitrile concentration in the water solution. On the other hand, UV-absorption spectral changes of uracil were found negligible at the different water–ACN ratios. Absorbance of calibration series of the aqueous solutions with increasing acetonitrile concentration (pure acetonitrile contained 15 mg l^{-1} of uracil) was measured by the Agilent VWD SL + detector at 254 nm. Values of the absorbance for each level of ACN concentration in the mobile phase fitted straight line with value of R^2 above 0.99.

3.2. Gradient profiles

Plots of the traced gradients mixed using 50-, 100-, and 250-μl syringes volumes, are shown in Fig. 4a, c, and e. S-shaped gradients of different steepness were created by successive sucking of adequate volumes of 5, 30, 55, and 80% (v/v) solution of acetonitrile (marked by 20 mg l^{-1} of uracil) in water according to Table 1. Gradient was delivered at the flow rate of 1 μl min^{-1} and 3 μl min^{-1}, respectively, where 250-μl syringe was used. Intensity of the turbulent mixing depends on the inner syringe diameter which also influences diffusion paths and the resulting gradient profile at all.

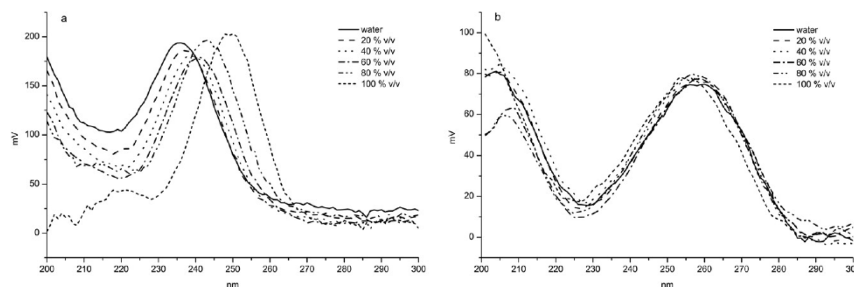

Fig. 3. UV spectra of (a) thiourea (b) and uracil at different water–acetonitrile ratio (measured by Agilent DAD 1200 series, optical path 6 mm; uracil and thiourea concentration was 1 mg l^{-1}, flow rate 10 µl min^{-1}).

When the 50-µl syringe was used, then linear gradients of volume from 8 to 30 µl were formed (Fig. 4a). Formation of a higher volume of linear gradient was difficult in this syringe because the measured plot became stepwise. Wider interval of gradient volume was achieved when 100-µl and 250-µl syringes were used. In these cases, linear gradients of volume from 15 to 70 µl (Fig. 4c), and from 50 to 200 µl (Fig. 4e) were formed.

The gradient profile is inevitably time dependent. Mobile phase gradient is prepared before the elution and the diffusion in the syringe barrel definitely affects it's profile. Therefore, the changes in gradient profile caused by diffusion were investigated by the series of experiments where the gradients of mobile phase created by sucking of constant volumes of 5, 30, 55, and 80% (v/v) solution of acetonitrile in water were pushed out from the syringe at different flow rates. Fig. 4b shows results obtained for the 50-µl syringe. The steepness of the S-shaped gradient observed at flow rate of 5 µl min^{-1} slightly decreases at lower flow rates while a linearity of the gradient profile improves. This trend became more evident when the 100-µl syringe is used (Fig. 4d). The effect of diffusion was the most apparent at 250-µl syringe. S-shaped gradient created in this syringe was transformed to convex when flow rates below 1 µl min^{-1} were applied (Fig. 4f). Based on these findings, the 100-µl syringe was found to be the most robust option for micro and nano column LC and gradient elution lasting 30 min may be considered at the flow rate from 0.4 to 2.5 µl min^{-1}.

3.3. *Performance of splitless gradient chromatographic system*

The suggested gradient system was firstly tested using a mixture of five alkylphenones containing valerophenone, hexanophenone, heptanophenone, octanophenone, and dodecanophenone each at the concentration of 20 µM in 5% (v/v) acetonitrile in water. Mobile phase gradient was prepared by successive sucking of 10, 5, and 10 µl of 30, 55, and 80% (v/v), solution of acetonitrile in water into the 100-µl glass syringe. Then, the sample volume of 0.5 µl was sucked into the sample loop. Separation was performed at the flow rate of 1 µl min^{-1} on capillary monolithic column placed in the column thermostat at a constant temperature of 40 °C. Absorbance was measured at 240 nm. The repeatability of the retention times was measured to test the uniformity of created gradients and obtained chromatograms are shown in Fig. 5 while the statistically evaluated data are presented in Table 2. Relative standard deviations (RSD) of retention times of 10 successive runs were up to 0.3%. Relative standard deviations of retention times obtained from 10-day experiment (3 runs per day) were up to 1.5%. We suppose that gradient generation repeatability is independent on the flow

Table 1
Volumes of mobile phases successively sucked into the syringe corresponding to linear gradients of different steepness showed in Fig. 4a, 4c, 4e.

% (v/v)	1	2	3	4	5	6	7	8	9
50-µl syringe (Fig. 4a)									
80	15	15	15	15	15	15	15	15	15
55	0	1	2	3	4	5	6	8	10
30	0	1	2	3	4	5	6	8	10
5	10	11	12	13	14	15	16	18	20

	1	2	3	4	5	6	7	8	9	10	11
100-µl syringe (Fig. 4c)											
80	20	20	20	20	20	20	20	20	20	20	20
55	3	4	5	6	8	10	12	14	16	18	20
30	3	4	5	6	8	10	12	14	16	18	20
5	13	14	15	16	18	20	22	24	26	28	30

	1	2	3	4	5	6	7	8	9
250-µl syringe (Fig. 4e)									
80	50	50	50	50	50	50	50	50	50
55	10	15	20	25	30	35	40	45	50
30	10	15	20	25	30	35	40	45	50
5	20	25	30	35	40	45	50	55	60

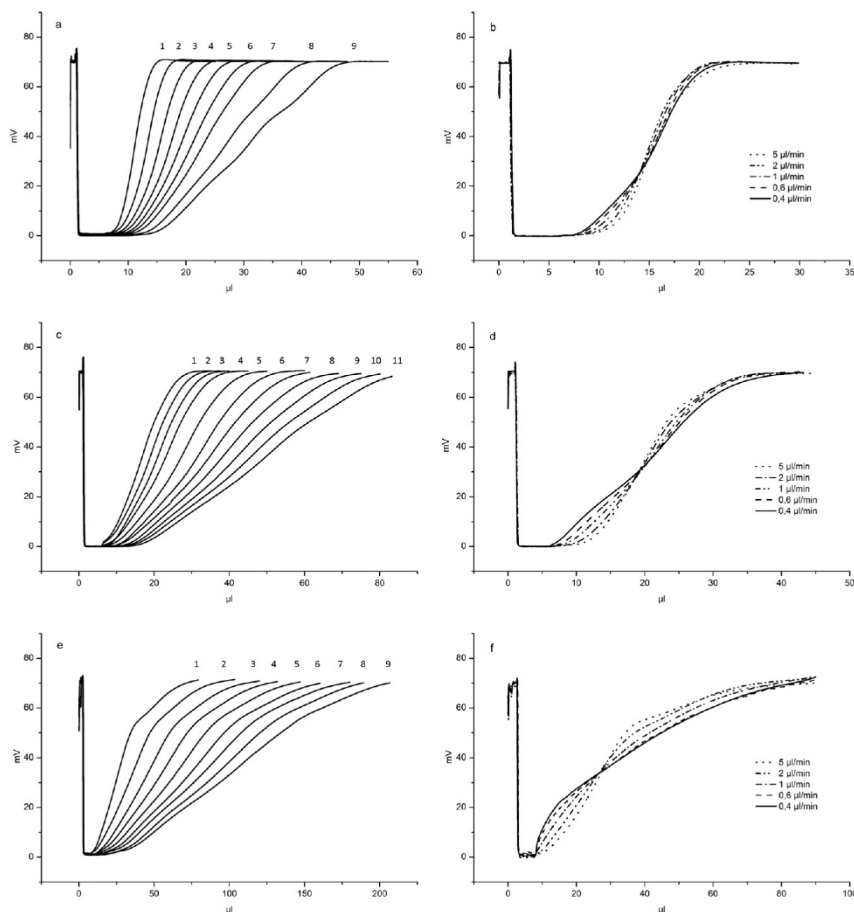

Fig. 4. Gradient profiles created by sucking of adequate volumes (Table 1) of 80, 55, 30, and 5% (v/v) solution of acetonitrile in water (100% acetonitrile contained 20 mg l^{-1} of uracil) into (a) 50-µl syringe, (c) 100-µl syringe, and (e) 250-µl syringe. Gradient was delivered at the flow rate of 1 µl min^{-1} and 3 µl min^{-1}, respectively, where 250-µl syringe was used. Gradient profiles were obtained at flow rates in the range from 5 to 0.4 µl min^{-1} using different syringes: (b) 50-µl syringe, (d) 100-µl syringe, and (f) 250-µl syringe.

rate during own analysis as the gradient generation takes under constant conditions just before elution. Thus, repeatability of the retention times at low flow rates is rather given by the syringe pump speed accuracy. Relative standard deviations of the retention times obtained at the mobile phase flow rate of 0.5 µl min^{-1} were up to 0.4%.

Sample delivery in the range of injection from 0.1 µl to 3 µl was tested as well. Adequate volumes of solution containing heptanophenone at the concentration of 10 µM in 5% (v/v) acetonitrile in water were injected. Model compound was eluted under isocratic conditions using 55% (v/v) of acetonitrile in water as the mobile phase. Other separation conditions stayed unchanged. Acquired values of peak area were statistically evaluated and relative standard deviations of peak area ($n = 5$) were about 5% and less. Fig. 6 demonstrates linearity of sample delivery within the given interval.

3.4. Separation of BSA

To demonstrate suitability of the chromatographic system to perform gradient elution of proteomic samples a solution of BSA tryptic peptides was injected and analyzed. Mobile phase gradient

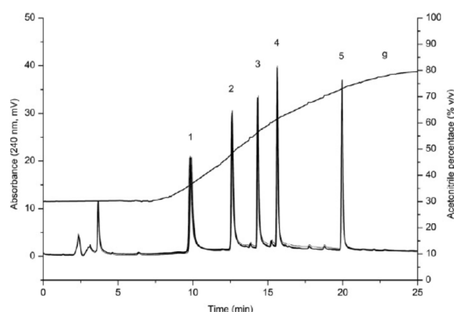

Fig. 5. Ten successive gradient separations of alkylphenones. Peak description: 1 = valerophenone, 2 = hexanophenone, 3 = heptanophenone, 4 = octanophenone, 5 = dodecanophenone, g = gradient track. Sample: 0.5 μl 2×10^{-5} M of each in 5% (v/v) acetonitrile–water solution. Column: Chromolith CapRod RP-18e 150 mm × 0.1 mm. Mobile phase gradient created by sucking of 10, 5, and 10 μl of 30, 55, and 80% (v/v) solution of acetonitrile in water. Flow rate: 1 μl min^{-1}. Column temperature: 40 °C.

Table 2
Repeatability of retention times in gradient elution.

Solute	Retention time (min)	Intra-day repeatability RSD (%, n = 10)	Inter-day precision RSD (%, 10 days, n = 30)
Valerophenone	9.84	0.31	1.39
Hexanophenone	12.62	0.17	0.87
Heptanophenone	14.34	0.15	0.63
Octanophenone	15.64	0.14	0.56
Dodecanophenone	19.96	0.07	0.49

was prepared by successive sucking of 15, 20, 20, and 20 μl of 5, 30, 55, and 80% (v/v) of acetonitrile in water (each solution contained 0.1% (v/v) of TFA) into the 100-μl glass syringe. The volume of 0.5 μl with total amount of 0.5 μg of BSA tryptic peptides in water was injected. The mobile phase flow rate was 0.5 μl min^{-1}. The temperature of the separation was maintained at 25 °C and detection was performed at 214 nm. Overlaid chromatograms are shown in Fig. 7. Relative standard deviation of the retention times (n = 8) of ten highest peaks were from 0.17 to 0.30%.

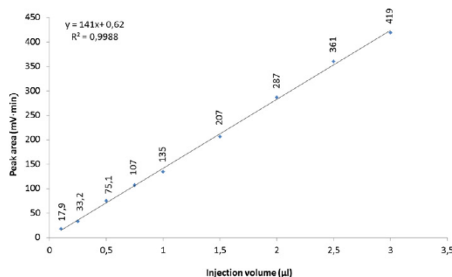

Fig. 6. The linearity of sample delivery; sample: 10 μM heptanophenone in 5% (v/v) acetonitrile–water solution. Column: Chromolith CapRod RP-18e 150 mm × 0.1 mm. Mobile phase: 55% (v/v) solution of acetonitrile in water. Flow rate: 1 μl min^{-1}. Column temperature: 40 °C.

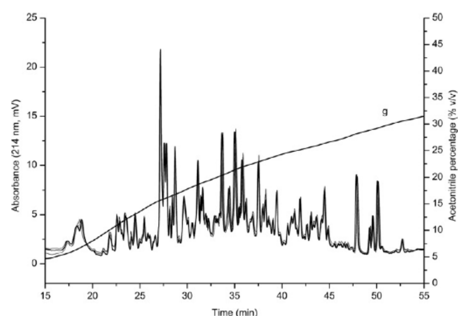

Fig. 7. Gradient separation of BSA tryptic digest – overlaid chromatograms of three successive runs. g = gradient track; sample: 0.5 μl of the digest (1 g l^{-1}). Column: Chromolith CapRod RP-18e 150 mm × 0.1 mm. Mobile phase gradient was created by sucking of 15, 20, 20, and 20 μl of 5, 30, 55, and 80% (v/v), respectively, solutions of acetonitrile in water (each contained 0.1% (v/v) of TFA). Flow rate: 0.5 μl min^{-1}. Column temperature: 25 °C.

4. Conclusion

The simple automated splitless gradient liquid chromatographic system for gradient elution on micro and nano columns was assembled and tested. System consists of the OEM syringe pump equipped with a glass syringe and a ten-port selector valve. Operation of the system is controlled by simple software compiled in labVIEW development environment allowing a direct adjustment of all volumes and flow rates. Gradient volume can be simply optimized by changing of sucked volumes of individual mobile phases. Uracil was found to be an excellent acetonitrile gradient tracer due to high molar absorption coefficient and negligible spectral shift over the entire interval of composition of water–ACN mixtures. Capability of suggested system to create linear gradient of various volume was verified using the 50-μl, 100-μl, and 250-μl glass syringes as mobile phase reservoirs, where different volumes of the individual mobile phases were sucked into the syringe barrel. Assuming the gradient elution on micro and nano columns the 100-μl syringe was found to be the most robust option. By utilization of this syringe it is possible to create linear gradient sufficient for 30-min gradient elution at the flow rate from 0.4 to 2.5 μl min^{-1}. Repeatability of gradient creation was tested using model mixture of five alkylphenones and values of RSD of retention times were lower than 0.3% which documents the high uniformity of gradient creation. Sample volumes in the range from 0.1 μl to 3 μl were injected with satisfactory precision (RSD of peak area <5%). Finally, the system was applied to a separation of BSA tryptic peptides to demonstrate suitability of the chromatographic system to perform the gradient elution of proteomic samples.

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3.4 Research Article IV

Simple power supply for power load controlled isoelectric focusing

Substantially high voltages are demanded to obtain separated zones of analytes in IEF analysis. Adapted power supplies for various modes of IEF are employed there due to differing requirements on voltage and applied power. Total applied power is especially important in strip format of IEF, apart from voltage which defines a width of separated zones at steady state of IEF analysis. Total applied power is directly responsible for Joule heat production as well as it determines time needed for completion of IEF analysis. Therefore, power load limitation prevents local overheating of separation bed while the highest feasible power load is maintained to finish separation as early as possible at given conditions. Although power load limitation is applied in a capillary and planar gel format where it is often a few watts, regulation from several tens to few hundreds milliwatts are needed for a strip format. First specialized power supplies capable of such task emerged only few years ago, but they are very expensive due to sophisticated technologies.

In the presented article a simple circuit of voltage multiplier was employed as a power supply for IEF. Features of voltage multiplier were advantageously used to set power load limit to 90 mW at peak which was optimized for separation bed made from nonwoven fabric of size 175 x 3 x 0.5 mm. Power load characteristics were then evaluated by a set of resistors with resistances common for strip format IEF. Finally, IEF analysis of model sample mixture containing colored p*I* markers was performed using the developed power supply. The power supply proved to be affordable alternative of commercial power supplies and it could be advantageously incorporated into a device integrating the power supply and

a focusing tray on one device thanks to a negligible intrinsic heat production.

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Simple power supply for power load controlled isoelectric focusing

Short Communication

Accepted for publication at Electrophoresis on December 16th 2013

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List of abbreviations:

CW VM, Cockcroft Walton voltage multiplier; **V/I**, voltage and current

Keywords:

Isoelectric focusing, power supply, voltage multiplier

Total number of words: 2752

Abstract

The power supply for isoelectric focusing (IEF) based on features of Cockcroft Walton voltage multiplier (CW VM) is described in this work. The article describes a design of the IEF power supply, its electric characteristics and testing by IEF analysis.

Circuit diagram of the power supply included two opposite charged branches (each consisting of 4 voltage doublers). The designed CW VM was powered by 230 V/50 Hz alternate current and it generated up to 5 kV and up to 90 mW at the output. Voltage and current characteristics of the power supply were measured by known load resistances in range from 10 kΩ to 1 GΩ which is common resistance range for IEF strip geometry. Further, the power supply was tested by a separation of a model mixture of colored pI markers using a 175 x 3 x 0.5 mm focusing bed. Automatically limited power load enabled analysis of samples without previous optimization of the focusing voltage or electric current time courses according to a sample composition. Moreover, the developed power supply did not produce any intrinsic heat and was easy to set-up with cheap and commonly available parts.

Isoelectric focusing is one of the major methods used for the protein separations [1]. Although solution phase [2] and capillary [3] modes of IEF are becoming more popular in proteomics, the 2D gel electrophoresis is still used and optimized in great number of labs [4]. However, IEF presents the most time consuming part of 2D gel electrophoresis or other multidimensional approaches and moreover, high IEF separation efficiency is crucial for final results. One of the main factors, which determines the width of focused analytes zones, is voltage applied during the focusing. In contrary, current is responsible for the transport of charged mass during IEF. In addition, Joule heat as a function of total applied power should be kept in mind. It can cause not only denaturation of analyzed proteins but the most importantly, it may lead to local overheating in a separation bed and therefore it can cause possible drying and decoupling of electric circuit, thus failing separation. Further, it is recommended to keep the temperature of the separation bed below 30 °C to prevent urea decomposition during IEF [5] and 20 °C proved to provide the optimal conditions for IPG IEF [6]. Joule heating is thereby the main factor which can contribute to longer times needed for completing IEF.

To overcome aforementioned problems, IEF should be run under the highest possible power conditions acceptable [5]. The control of the IEF progress by using a power load limitation, which can be altered during focusing run, enables to cut time needed for IEF completion to a minimum. Furthermore, optimization of the voltage time course is unnecessary owing to the power load limitation. This approach is well known in planar gel IEF, but concerning small geometry of IPG strip and other similar small separation beds, leakage of current becomes a problem since it can interfere with power supply electronics and hence it can prevent from a proper power control.

A possible solution to minimize Joule heat is programming of the voltage applied to the separation bed during IEF. Classical way is a gradual voltage increase [7]. Another possibility is application of current limits, or combination of both: voltage time course programming and limiting of the current. Some manufacturers recommend to run IPG strips with default current limit of 50 µA per strip in order to keep the running temperature low. Moreover, to ensure the highest reproducibility of IEF the integral volt x hours should be kept the same between individual separations [5]. Basically, it is possible to devise and optimize a universal time course setting for voltage/current leading to reasonable separation time when analyzing samples of similar composition, concerning mainly matrix and concentration of salt and analytes. On the other hand, if the samples differ in mentioned properties (especially salt concentration), it is advantageous to use limited current in the universal method to prevent possible overheating [5]. As a result, one must expect increased IEF run time of samples with high concentration of salts.

Recently, Agilent technologies and Bio-Rad proposed their new state of the art instruments: 3100 OFFGEL Fractionator (0.5 – 10 kV, 0 – 150 µA, 0 – 1 W) and PROTEAN® i12™ IEF System (0.5 V - 10 kV, 0 – 100 µA, 0 – 1 W) respectively. Both instruments are capable to individually control each focusing line.

In addition, there are two other systems integrating focusing cell and power supply. Ettan™ IPGphor™ 3 Isoelectric Focusing System from GE Healthcare Life Sciences and IEF100 from Hoefer can reach up to 10 and 12 kV at output respectively and they can use current limitation. However, IPG strips in those two instruments are not controlled individually and moreover Ettan™ IPGphor™ 3 cannot limit total applied power. Note that all given information were taken from particular instruments manuals available at websites of manufacturers. Apart from this four specialized IPG power supplies, there are many universal electrophoretic power supplies applicable for standalone IPG focusing trays. Although those power supplies can handle IEF by programing V/I time course, they are in general, due to sophisticated electronics and controlling software, quite costly and sometimes bulky. Therefore, development of alternative power supplies was encouraged [4]. For overview of advances of the development of commercial and laboratory-built high-voltage power supplies employed for capillary and microchip electrophoresis see [8].

This article offers design of simple high-voltage IEF power supply with power load time course limitation. The power supply is based on features of classic multistage diode/capacitor voltage multiplier, known as Cockcroft Walton voltage multiplier, (CW VM). It is one of the cheapest and most popular ways of generating high voltages at relatively low currents. CW VM is used in wide range of electrical appliances including photo flashes, photocopiers, x-ray sources, photomultipliers, and many others. Power supply based on CW voltage multiplier was also used in capillary zone electrophoresis previously [9,10] as means of reaching 100 kV. CW VM output voltage is highly dependent on the current load. When high amount of current is drawn from circuit, voltage rapidly decreases. The result of this effect is approximately hyperbolic shape of voltage-current characteristics, which can serve well for charging the output electrodes to high voltages at roughly constant charging power. Above mentioned characteristics make the CW multiplier suitable for utilization in IEF which requires low power controlled load.

On the basis of those findings, the CW VM power supply for IEF was constructed, its electric properties examined, and it was tested with model IEF run. The power supply circuit was designed to provide similar voltage time profile during IEF run as in the voltage program previously used and optimized for focusing in narrow strip geometry [11].

For the testing of the new power supply we used following chemicals: 2-amino-2-methyl-1,3-propanediol (Ammediol), 2,2-Bis(hydroxymethyl)-2,2',2''-nitrilotriethanol, creatinin, butan-1-ol, ethylene glycol, Glycyl-glycine, Glutamic acid, HEPES, histidin, 4-(2-Hydroxyethyl)-1-piperazinepropanesulfonic acid, imidazole, N-Methyl-D-glucamine, Patent blue V sodium salt, sucrose, and Tris from SIGMA-ALDRICH (St. Louis, MO). Colored pI markers were developed and prepared in authors' laboratory mainly by diazotation and/or Mannich reaction [12–16]. Distilled water was prepared fresh in laboratory.

All IEF analyses were performed on the device which was thoroughly described in [11]. Briefly, IEF was run in separation bed in form of 175 x 3 x 0.5 mm nonwoven fabric strip placed in lab-made plastic focusing tray. The tray was clipped down with pin wire electrodes (end of the anode was made from carbon) on a box with the CW VM power supply installed inside. The assembled device was connected to 230 V / 50 Hz AC power line.

As was mentioned in the introduction, the design of the power supply took advantage of CW VM scheme. The diodes rated for 1 A / 1000 V and 10, 15, 22, 33, 47, 68, and 100 nF capacitors rated for 250 V AC were used for construction of the power supply. Those particular parts cost less than 10 cent € per item and complete set resulted in no more than 10 € including on/off switch, necessary connection wires and supporting plate. All parts were connected according to the electric circuit diagram shown in Figure 1A. Two power supplies set up in parallel are shown in Figure 1B. After assembling, outputs of the two power supplies were connected to electrodes and power supplies were mounted into the box holding the two IEF trays.

Before the beginning of each IEF run, a 0.5 mm thick sheet of nonwoven fabric Novoline 80 $g.m^{-2}$ (Polytex s.r.o., Malé Svatoňovice, Czech Republic) was cut into 3 mm wide and 175 mm long strip. Then it was placed and aligned in the IEF tray between electrodes.

Consequently, the model mixture of colored pI markers along with simple buffers and ampholytes was prepared as described earlier [11]. Finally, the mixture was loaded onto the fabric strip and the power supply was switched on and the ammeter was connected between the power supply and the electrodes. The current was registered during the whole IEF run. A photo of resulting zones of colored pI markers was taken after the steady state of IEF was reached.

The power supply was calibrated by loading of the circuit with a set of resistors of known resistances. The range of the resistances covered 5 orders of magnitude, beginning with 10 kΩ. The range of the resistances used for load calibration is typical for a great majority of IEF strip runs. Digital micro-ammeter was used to register a current. After calibration with the whole set of resistors, a graph was generated with logarithm of the resistance at the X axis and the corresponding current on the Y axis. The output voltage and power were calculated using Ohm's law and both values were plotted into the same graph. According to obtained voltage and current (V/I) characteristics, capacities of particular capacitors in the voltage multiplier were optimized to meet desired power limit of approximately 90 mW and the calibration was repeated. After the optimization, the final V/I characteristics graph was produced (see figure 2). From the graph, it is clearly visible that the applied power increased from 25 mW to 88 mW at the maximum while the resistance increased from 10 kΩ to 1 GΩ. The power load limitation of 25 mW at low resistances was important to avoid excessive Joule heat production in the focusing bed during the IEF desalting step. It should be emphasized that suggested results are valid only for 230 V / 50 Hz AC power line feed. A transformer must be used to reach given qualities in case of different power line characteristics.

After the optimization of the power supply V/I characteristics, IEF run of model mixture powered by suggested power supply was monitored. The micro-ammeter was connected into the electrophoretic circuit and the current was registered during IEF of simple background buffers mixed with colored pI markers. A resistance time course was calculated from the power supply characteristics with usage of Microsoft Office™ Excel™ interpolate function freely available in add-on XlXtrFun™. Corresponding voltage and power were subsequently calculated and all mentioned quantities were plotted into a graph (see figure 3A). As one can see, voltage and current started from 89 V and 280 μA and they reached 5 kV and 7.5 μA after 14 hours of focusing with the time course very similar to the one programmed previously [11]. The resistance of the strip increased throughout the whole IEF run in the range expected for the used tray geometry (figure 3A). The graph showed that power load of developed power supply was automatically regulated during the IEF run. Power load was advantageously kept low at the beginning of the IEF and further it raised to maximum at 88 mW, 2.6 kV and 33 μA (figure 3A). The steady state of IEF was indicated by focused zones of colored pI markers (figure 3B). Two parallel focusing trays, which had been run independently from each other by using two separate CW VM power supplies, are shown in this figure. Similar pI marker zones patterns were observed in both cases.

To summarize, we described the design of a simple power supply which is able to maintain desired power limit over the load resistance range of five orders of magnitude. This feature enables a self-regulation of IEF run with the minimal possible separation time. The power supply design has a perspective for utilization in IEF of small geometries where a power load limitation with common programmed power supplies becomes tricky. Furthermore, the power supply does not produce any heat due to the absence of resistors and therefore, it can be integrated within the device in which IEF proceeds. Moreover, the suggested power supply is easy to set-up and the used parts are cheap so it can be applied in large number for parallel IEF runs.

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Conflict of interest statement

We declare no financial/commercial conflict of interest.

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Figures

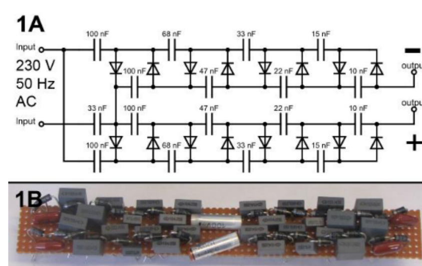

Figure 1

1A – Power supply circuit diagram

Diodes are rated for 1 A / 1000 V, capacitors with capacities designated in the figure are rated for 250 V AC. On the left are contacts to 230 V 50 Hz AC and on the right are electrode outputs.

1B – Photo of two assembled parallel placed CW VM power supplies

Two pairs of input contacts for 230 V 50 Hz AC are in the middle. Positive and negative branches of both power supplies stretch to the opposite ends along the whole length of the support plate. Two anode outputs are on the left side and two cathode outputs are on the right side.

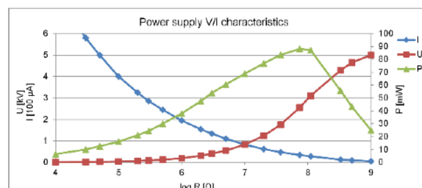

Figure 2

V/I characteristics of the suggested power supply

The power supply output electrodes were loaded by resistors of known resistance. The measured current was used for voltage and power calculation.

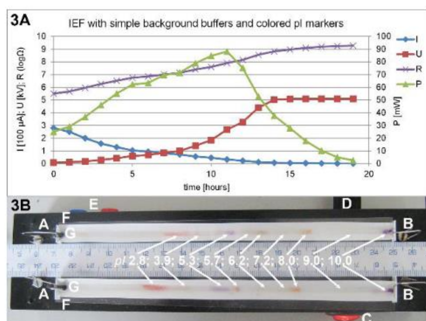

Figure 3

3A – Electrical characteristics time course of IEF run powered by CW VM power supply

The registered current was used for calculation of corresponding time course of resistance, voltage and power

3B - Image of two parallel steady state IEF runs of the model sample mixture powered by two independent CW VM power supplies

A – anodes; B – cathodes; C – on/off switch; D – connection to AC power line; E – contacts for connection to micro-ammeter; F – focusing tray with nonwoven fabric strip; G – focused zones of pI markers (red, 2.8; orange, 3.9; lavender, 5.3; yellow, 5.7; red, 6.2; yellow 7.2; orange, 8.0; yellow, 9.0; violet, 10.0; for details see [11])

4 CONCLUSIONS

Biological matrices represent set of samples which is usually hard to analyze with only one separation method. Therefore, new approaches including two or more synergic connections of separation methods are emerging in the field. According to the literature research, IEF is commonly employed in the first dimension followed in most cases by RPLC. Similarly, we proposed two 2D methods composed of either a free-flow IEF or a solution IEF in the first dimension with a capillary RPLC employed for the second dimension.

The first research paper proved that DF-IEF can be used for a fractionation or peptides without external carrier ampholytes forming pH gradient. Moreover, DF-IEF device was capable to separate a heterogeneous cluster of CMP glycosylated peptides. Therefore, similar bulky matrices should be efficiently separated using DF-IEF and thanks to its continuous character, high volumes of samples could be processed and fractionated. Moreover, use of nonwoven fabric was beneficial due to easy modification into desired shape and natural filtration effect in net of fibers. On the contrary, filtration can sometimes result into clogging of separation bed. Finally, separation in the second dimension enabled to resolve both mixtures into individual peaks of peptides/proteins, therefore information about mixture composition was revealed.

The experience with nonwoven fabric in IEF was further used in strip format of IEF in the second research paper. Due to transferring into batch IEF system, a protocol for fraction extraction needed to be devised. Optimized system was used for fractionation of whey. Moreover, the system was used in IEF trapping mode to improve separation resolution. Results proved that the technique was able to almost completely

separate CMP from two major proteins contained in the whey mixture. RPLC-DAD-UV was again used in second dimension for identification of whey components.

Furthermore, a novel approach to automated RPLC mobile phase gradient formation was presented. The suggested instrument was capable to separate analytes including peptides with high repeatability while using sub-microliter volumes of samples. Later, the system proved that it could be employed for two-dimensional analysis. This was realized in year 2013 where mentioned system was used for 2D analysis of peptide mixture using electrically decoupled CIEF in the first dimension coupled to splitless nano RPLC system by usage of trap columns. Data were presented recently at international conference HPLC 2013 in Amsterdam.

IEF separation time can be shortened when maximal possible power (considering excessive Joule heat production) is applied to the separation bed. Therefore, an alternative to established power supplies was suggested recently. Invented power supply electrical characteristics were evaluated and system was tested with a model IEF run. It was proved that power supply adapted to precise power load limit of 90 mW at peak was capable to focus analytes effectively.

I showed that IEF and RPLC are two efficient fractionation techniques which are easily incorporated into two-dimensional separation schemes. Furthermore, those techniques can be easily modified to match required application. In this work I have proved, that those techniques are ready to be applied for two-dimensional separation of proteins and peptides from biological matrices.

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APPENDIX

Curriculum vitae

PERSONAL DETAILS

Name, title:	**Filip Duša, Mgr.**
Date of Birth:	31. 12. 1985
Address:	Ke Skalce 336, Fryšták, 76316, Czech Republic
Nationality:	Czech
Telephone:	+420 775 700 048
E-mail:	filip.dusa@gmail.cz

EDUCATION

2010 – present	Ph.D. study, Department of Biochemistry, Faculty of Science, Masaryk University, Specialization: Biochemistry
2008 – 2010	Master's degree, Department of Biochemistry, Faculty of Science, Masaryk University, Specialization: Biochemistry
2005 – 2008	Bachelor's degree, Department of Biochemistry, Faculty of Science, Masaryk University, Specialization: Biochemistry
2001 – 2005	Grammar school, Gymnázium Ladislava Jaroše, Holešov

Working experience

2010 – present

Ph.D. thesis - Multidimensional separation methods in analysis of complex biological mixtures (supervisor: RNDr. Vladislav Kahle, CSc., IACH, ASCR, Brno)

2008 - 2010

Master's thesis – Capillary separation methods in analysis of metabolome and complex biological matrixes (supervisor: RNDr. Vladislav Kahle, CSc., IACH, ASCR, Brno)

2007 - 2008

Bachelor's thesis - Study of copper binding proteins and metallothionien in samples from patients with cancer (supervisor: prof. Ing. René Kizek, Ph.D., MZLU, Brno)

SKILLS

Laboratory experience:

reversed phase liquid chromatography; isoelectric focusing in liquid phase, gel, and capillary; SDS-PAGE, tryptic digestion of proteins; protein labeling; laser induced fluorescence, capillary zone electrophoresis

One day workshop with certificate - Albertov Comprehensive Days 2011 (February 9th 2011) focused on multidimensional and comprehensive liquid chromatography and its aplications

Language - English: FCE Cambridge certificate - level B2 - (June 2013)

Computer: MS WINDOWS, MS OFFICE, Origin, Google SketchUp, Adobe Photoshop, Adobe Acrobat Professional, Inkscape, Simul 5.0 (software for simulation of electrophoretic analysis)

Driving license: group B

List of Publications

Articles

Filip Duša, Karel Šlais (2013). *Simple power supply for power load controlled isoelectric focusing.* Electrophoresis, Accepted on December 16th 2013

Jozef Šesták, Filip Duša, Dana Moravcová, Vladislav Kahle (2013). *Simple automated liquid chromatographic system for splitless nano column gradient separations.* Journal of chromatography A, 1276, 26–32. doi:10.1016/j.chroma.2012.12.020

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Filip Duša, Jana Křenková, Dana Moravcová, Vladislav Kahle, Karel Šlais (2012). *Divergent-flow isoelectric focusing for separation and preparative analysis of peptides.* Electrophoresis, 33(12), 1687–1694. doi:10.1002/elps.201100587

Oral presentations

Filip Duša, Karel Šlais. *Micropreparative solution isoelectric focusing of peptides and proteins in nonwoven strip.* CECE Junior 2013, Brno, Czech Republic, Proceedings CECE 2012, 66-68, ISBN: 978-80-904959-1-3

Filip Duša, Dana Moravcová, Vladislav Kahle, Karel Šlais. *Využití izoelektrické fokusace v rozbíhavém toku a kapilární reverzně-fázové kapalinové chromatografie při separaci modelové směsi peptidů.* Monitorování cizorodých látek v životním prostředí XIII. Luka nad Jihlavou, 2011, 35-43, ISBN 978-80-7395-450-5

Posters

Filip Duša, Karel Šlais. *New isoelectric focusing power supply based on features of voltage multiplier.* CECE Junior 2013, Brno, Czech Republic, Chemické listy, 107, s3, 358-359, ISSN 1213-7103

Filip Duša, Jozef Šesták, Dana Moravcová, Josef Planeta, Vladislav Kahle. *Simple two-dimensional separation platform for peptide analysis*. HPLC 2013, Amsterdam, Netherlands, Abstract Book, 293, ISBN 978-83-7780-440-447

Filip Duša, Karel Šlais. *Micro fractionation of proteins and peptides by solution phase isoelectric focusing in nonwoven fabric strip.* ISC 2012, Torun, Poland, Abstract book, 293, ISBN 978-83-7780-440-7

Filip Duša, Karel Šlais. *Purification of caseinomacropeptide by novel solution phase isoelectric focusing device*. CHIRANAL 2012, Olomouc. CHEMICA 50S. 88-89 ISBN 978-80-244-3115-4 (**Best poster award**)

Filip Duša, Jozef Šesták, Dana Moravcová, Marie Horká, Vladislav Kahle. *Splitless gradient nanocolumn liquid chromatographic system for proteomic purposes*. 36th ISCC 2012, Riva del Garda, Italy, Abstract Book. 265

Filip Duša, Dana Moravcová, Vladislav Kahle, Karel Šlais. *Divergent flow isoelectric focusing of whey caseinomacropeptide*. CECE 2011 Brno. 38, ISBN 978-80-904959-0-6

Filip Duša, Jana Křenková, Dana Moravcová, Vladislav Kahle, Karel Šlais. *Pre-Separation of High Volume Biological Samples using Divergent Flow Isoelectric Focusing*, HPLC 2011 Budapest Symposium, Book of Abstracts. 145, ISBN 978-963-89335-0-8

Filip Duša, Marie Vykydalová, Dana Moravcová, Vladislav Kahle, Karel Šlais. *Two-dimensional separation of peptides combining divergent flow isoelectric focusing and capillary liquid chromatography*. Microscale Bioseparations 25th MSB 2010, 107-108, ISBN 978-80-254-6631-5

Filip Duša, Ivo Fabrik, Dalibor Húska, Vojtěch Adam, František Jelen, Libuše Trnková, René Kizek. *Electrochemical detection of ceruloplasmin.* Electrochemistry of Nucleic Acids and Proteins 2008, Book of Abstracts, 81