New analytical tools combining gel electrophoresis and mass spectrometry

THÈSE N^{O} 6331 (2014)

PRÉSENTÉE LE 25 SEPTEMBRE 2014

À LA FACULTÉ DES SCIENCES DE BASE
LABORATOIRE D'ÉLECTROCHIMIE PHYSIQUE ET ANALYTIQUE
PROGRAMME DOCTORAL EN CHIMIE ET GÉNIE CHIMIQUE

ÉCOLE POLYTECHNIQUE FÉDÉRALE DE LAUSANNE

POUR L'OBTENTION DU GRADE DE DOCTEUR ÈS SCIENCES

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Suisse
2014

To my parents and my husband…

Моим любимым родителям и мужу посвящаю…

Acknowledgement

First of all, I would like to thank my thesis supervisor, my professor, boss and just a good person Professor Hubert Girault for all the help, support, advice, creative ideas and new knowledge that he has given me during my PhD studies. Thank you very much for believing in me and giving me such an opportunity to become a LEPA member. I have had an amazing experience working in Hubert's group with so many talented and interesting people.

I also would like to thank, Prof. Yury Tsybin, Dr. Joel Rossier and Prof. Serge Rudaz for accepting to be jury members for my PhD defense. I really appreciate the time the jury members gave reading my thesis. Moreover, I want to thank Prof. Anne-Clémence Corminboeuf for being the president of the jury.

I have to acknowledge my collaborators who contributed a lot to my work: Dr. Fernando Cortés-Salazar, for supporting and providing me with useful advices during my first year of PhD, Dr. Liang Qiao for his kind assistance in chemical and vital questions and for helping me with mass spectrometry analysis, Romain Sartor, Yu Lu, Dmitry Momotenko for sharing with me not only the office but also the knowledge about finite element modeling, Julien Maillard for providing me with biological samples, Dr. Christophe Roussel for useful suggestions. Moreover, I wish to express my deep thanks to all other former and current members of LEPA for being friendly and kind with me, especially Dr. Yu Lu, Dr. Kathryn Ellen Toghill, Dr. James Thomas Stockmann, Evgeny Smirnov, Dr. Micheal Scanlon. I also warmly would like to thank Madame Maria Szuman, our former secretary, for her great job, for her big heart and great support. At the beginning of the stay she was solving all the problems with apartment, administrative work, without her help my stay at the beginning won't be so pleased. Madame Valérie Devaud, our technician who was always there for any technical questions, Madame Patricia Byron-Exarcos, our secretary, just a wonderful, kind person, who brings so many positive emotions and optimism to the group and Anne-Lene Odergaard for providing me with useful information before and during my PhD.

I also wish to thank the work of technicians from Mechanical Workshop and Electronic Workshop of Institute of Chemical Sciences and Engineering at EPFL, especially André Fattet and Frédéric Gumy.

I also thank all my friends, colleagues and my diploma supervisor from Saint-Petersburg, Russia for your “long-distance” support, for not forgetting me and for living through all the ups and downs with me.

And finally, but most importantly, I would address my special thank to my family, to my grandparents, parents, Larisa and Alexey Tobolkiny for everything that you all did for me and I thank my only love, my everything, my husband, Alexey Kovgar, whom I meet during my first year of PhD. Without you, your love, support, advices and motivation this work would not be completed. I dedicate my thesis to the most important people in my life.

Abstract

Proteomics has been one of the main projects challenging biological and analytical chemists for many years. The separation, identification and quantification of all the proteins expressed within biological systems remain the main objectives of proteomics. Due to sample complexity, the development of fractionation, separation, purification and detection techniques that possess appropriate resolution to separate a large number of proteins, as well as being sensitive and fast enough for high throughput protein analysis are required.

The objective of this thesis was to develop new separation strategies for protein/peptide fractionation using gel electrophoresis and its further detection by mass spectrometry analysis.

A multi-electrode set up based on OFFGEL electrophoresis was developed. The objective was to provide a more efficient application of the electric field for fast and improved protein separation. The results showed that using a multi-electrode setup provides not only a higher protein separation but also better protein collection efficiency.

Electrophoretic separation using an ultra narrow pH gradient (UNPG) gel was adapted for a three-well OFFGEL device for fast sample purification and desalting. Purification of an *E.coli* extract was applied to demonstrate that electrophoretic separation with UNPG gels provides an efficient strategy for fast purification of complex biological samples and can be utilized as a preparative technique in proteomics. UNPG gels were also used to separate charge molecules taking place in a new electro-elution device. The molecules were washed from the gel surface by an aqueous buffer and collected for further analysis by mass spectrometry. The electro-elution device provides a fast approach that avoids time-consuming steps of extraction from the polyacrylamide gel.

An electrostatic spray ionization (ESTASI) mass spectrometry technique was developed in our group to ionize sample solutions on different substrates. ESTASI has been here coupled to isoelectric focusing and demonstrated to be a powerful tool, which can improve the detection sensitivity compared to standard visualizing

protocols. Paper-ESTASI-MS was developed and applied for rapid perfume analysis of six authentic fragrances as a high throughput and sample preparation-free method.

ESTASI was also applied for quantitative analysis of caffeine in different beverages using a standard addition strip. The results were compared to classical standard addition methods for MS or LC. It was shown that the strip strategy could be utilized for fast and accurate food analysis and quality control. Desalting and direct analysis of samples from ZipTip by ESTASI-MS has been demonstrated.

Keywords: proteomics, gel electrophoresis, isoelectric focusing, mass spectrometry, electrospray ionization, matrix-assisted laser desorption ionization, electrostatic spray ionization

Résumé

La protéomique a représenté l'un des principaux défis pour les biochimistes et les chimistes analyticiens durant de nombreuses années. La séparation, l'identification et la quantification de toutes les protéines exprimées dans les systèmes biologiques représentent les principaux objectifs de la protéomique. En raison de la complexité de chaque échantillon et afin de permettre des analyses de protéines à haut débit (high throughput), il est nécessaire de développer des techniques de fractionnement, de séparation, de purification et de détection possédant une résolution suffisante pour séparer un grand nombre de protéines, tout en étant suffisamment sensible et rapide.

L'objectif de cette thèse est de développer de nouvelles stratégies de fractionnement de protéines/peptides basées sur l'électrophorèse sur gel et associées à une détection ultérieure par spectrométrie de masse.

Un système multi-électrode reposant sur le principe de l'électrophorèse OFFGEL a été développé. L'objectif étant de rendre plus efficace l'application du champ électrique afin d'obtenir une séparation de protéines rapide et améliorée. Les résultats ont montré que l'utilisation d'une configuration multi-électrodes permet non seulement une meilleure séparation des protéines, mais aussi une meilleure efficacité de collecte des protéines.

Le principe de la séparation électrophorétique sur un gel caractérisé par un gradient de pH extrêmement réduit (UNPG, pour *U*ltra *N*arrow *p*H *G*radient) a été adapté à un dispositif d'électrophorèse OFFGEL à trois puits dans le but d'obtenir une purification de l'échantillon et un dessalage rapide. La purification d'un extrait de *E. Coli* a été utilisée pour démontrer que la séparation électrophorétique avec des gels UNPG représente une stratégie efficace pour la purification rapide d'échantillons biologiques complexes et qui peut être utilisée comme une technique de préparation en protéomique. Les gels UNPG ont également servis pour séparer des molécules chargées, dans un nouveau système d'électro-élution. Les molécules sont extraites de la surface du gel par une solution tampon et collectées pour une analyse ultérieure par spectrométrie de masse. Le dispositif d'électro-élution fournit donc une approche

rapide en permettant d'éviter les étapes fastidieuses d'extraction des protéines/peptides du gel de polyacrylamide.

La technique de spectrométrie de masse d'ionisation par nébulisation électrostatique (ESTASI) qui a été développé dans notre groupe permet d'ioniser la solution contenant un échantillon sur différents substrats. Dans ce travail, la technique ESTASI, lorsqu'elle est couplée à la focalisation isoélectrique, s'est montrée un outil puissant, capable d'améliorer la sensibilité de détection par rapport aux protocoles standards de visualisation. En particulier, la méthode papier-ESTASI-MS a été développée et appliquée pour une analyse rapide de parfums. Six échantillons de parfums authentiques ont été testés pour démontrer la faisabilité de cette méthode, permettant des analyses haut débit et n'exigeant aucune préparation.

La technologie ESTASI a également été appliquée à l'analyse quantitative de la caféine dans différentes boissons par comparaison avec des gouttes de concentration progressive (additions standard) déposées sur une bandelette. Les résultats ont été comparés à ceux obtenus à partir des méthodes d'addition standard classiques dans la MS et la LC. Il a été montré que la stratégie des bandelettes pourrait être utilisée pour une analyse rapide et précise d'aliments et pour le contrôle de qualité. Le dessalage et l'analyse directe d'échantillons de ZipTip par ESTASI -MS a été démontrée.

Mots-clés: protéomique, électrophorèse sur gel, focalisation isoélectrique, spectrométrie de masse, ionisation par électronébulisation, désorption-ionisation laser assistée par matrice, ionisation par nébulisation électrostatique

List of abbreviations

Abbreviation	Meaning
ACN	Acetonitrile
Ang I	Angiotensin I
AP	Atmospheric pressure
APS	Ammonium persulfate
BSA	Bovine serum albumin
CA	Carrier ampholytes
CHCA	α-cyano-4-hydroxycinnamic acid
CE	Counter electrode
CE	Capillary electrophoresis
CID	Collision induced dissociation
CRM	Charge residue model
CYP450	Cytochrome P450
Cys-CAM	Cysteine carbamidomethylation
DC	Direct current
DESI	Desorption electrospray ionization
DHB	2,5-dihydroxybenzoic acid
DTT	1,4-dithiothreitol
ECD	Electron capture dissociation
EESI	Extractive electrospray ionization
ETD	Electron transfer dissociation
ESI	Electrospray ionization
ESTASI	Electrostatic spray ionization
ExPASy	Expert protein analysis system
FEM	Finite Element Model
FID	Flame ionization detection
FT-ICR	Fourier transform ion cyclotron resonance
GC	Gas chromatography
HPA	3-hydroxypicolinic acid
HPLC	High-performance liquid chromatography
HV	High voltage
IAA	Iodoacetamide
ICR	Ion cyclotron resonance
IEM	Ion evaporation model
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
IT	Ion trap
LC	Liquid chromatography
LDI	Laser desorption/ionization

LOD	Limit of detection
M.W.	Molecular weight
m/z	Mass-to-charge ratio
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
OGE	OFFGEL electrophoresis
PDEs	Partial differential equations
p*I*	Isoelectric point
PMF	Peptide mass fingerprinting
PTMS	Post translational modification
RPC	Reversed phase chromatography
S/N	Signal-to-noise ratio
SA	Sinapinic acid
SCX	Strong cation exchange chromatography
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
2D-PAGE	Two-dimensional gel electrophoresis
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TIC	Total ion current
TOF	Time of flight

List of symbols

Symbol	Quantity	Units
c_i	local concentration of species *i*	mol•m^{-3}
D_i	diffusion coefficient of species *i*	m^2•s^{-1}
E	electrical field strength	V•cm^{-1}
ε_o	vacuum permittivity	F/m
ε_r	relative permittivity	none
ϕ	electrical potential	V
F	Faraday constant	=96485 C•mol^{-1}
i	current	A
K_i	ionization constant of the ionizable group	none
M	molecular weight	kg•mol^{-1}
R	gas constant	= 8.34151 J•mol^{-1}•K^{-1}
T	temperature	K
t	time	s
$\tilde{u}$	electrochemical mobility	m^2 •J^{-1} •s^{-1}
u	electric (or electrophoretic) mobility	m^2 •J^{-1} •s^{-1}
V	voltage	V
V_m	migration velocity	cm•s^{-1}
z_i	charge number of species *i*	none
σ	electrical conductivity	S•m^{-1}

Table of Contents

Chapter I. Introduction 1
1. Proteomics 2
1.1. Proteome and genome 2
1.2. Strategies in proteome research 3
1.3. Techniques in proteome research 5
2. Gel-based separation approaches 7
2.1. Isoelectric focusing (IEF) 8
2.1.1. Immobilized pH gradient (IPG) gel 13
2.1.2. OFFGEL electrophoresis (OGE) 16
2.2. SDS-PAGE electrophoresis 18
2.3. Two-dimensional (2D) gel electrophoresis 20
3. Mass spectrometry 22
3.1. Matrix-assisted laser desorption ionization 22
3.2. Electrospray ionization mass spectrometer 25
3.3. Ambient ionization mass spectrometry 27
4. Thesis outline 29
References 32

Chapter II. Segmented Field OFFGEL® Electrophoresis 45
1. Introduction 46
2. Materials and methods 48
2.1 Materials 48
2.2 OFFGEL electrophoresis using two- and multi-electrode setups 48
2.3 Soluble E. coli protein extract preparation 49
2.4 MS analyses 50
2.5 Finite element simulations 50
3. Results and discussion 51
3.1 Numerical simulation of protein migration and electric field distribution in the OFFGEL device 51
3.2 OFFGEL electrophoresis with a multi-electrode setup 54
3.3 OFFGEL fractionation of E.coli extract 61
4. Conclusions 64
Appendix 2.0 65
References 67

Chapter III. Proteins/peptides purification by three-well OFFGEL electrophoresis with immobilized ultra narrow pH gradient gels 69
1. Introduction 70
2. Materials and methods 72
2.1 Materials 72
2.2 UV-Vis spectroscopy 72
2.3 Soluble E. coli protein extract preparation 72
2.4 Tryptic digestion 73
2.5 Electrophoretic purification with immobilized UNPG gels 73
2.6 MALDI-MS analysis 74
2.7 LC-MS/MS analysis 74
2.8 Simulations 75
3. Results and discussion 76
3.1 Principle: electrophoretic separation with immobilized UNPG gels 76
3.2 Purification on UNPG gels 78

3.3 Sample desalting on UNPG gels *79*
3.4 Purification of E. coli extract on UNPG gels *81*
3.5 Purification of digested E. coli extract followed by LC-MS/MS analysis *82*
4. Conclusions 85
Appendix 3.0 ..... *86*
References 93

Chapter IV. Electro-elution device for protein/peptide purification using ultra narrow pH gradient gels 95
1. Introduction 96
2. Material and Methods 98
2.1 Chemicals & Materials *98*
2.2 BSA digestion *99*
2.3 Protein/peptide purification by electro-elution device *98*
2.4 MS analysis *100*
3. Results and discussion 100
3.1 Elution of protein mixture for MALDI-MS analysis *102*
3.2 Elution of protein with online ESI-MS analysis *104*
3.3 MALDI-MS detection of peptide mixture fractionated by an electro-elution device ... *106*
4. Conclusions 108
References 109

Chapter V. Coupling Isoelectric Focusing Gel Electrophoresis to Mass Spectrometry by Electrostatic Spray Ionisation 111
1. Introduction 112
2. Materials and methods 115
2.1 Materials *115*
2.2 BSA digestion *115*
2.3 Soluble Escherichia coli protein extract preparation *115*
2.4 Gel electrophoresis *116*
2.5 Gel on a plastic support drilled with holes *116*
2.6 Gel-ESTASI-MS *116*
2.7 Gel based IEF-MALDI-MS *117*
2.8 OFFGEL IEF-ESI-MS *118*
2.9 Numerical simulation *118*
3. Results & Discussion 119
3.1 Electrostatic spray ionisation of peptide or protein from polyacrylamide gel *119*
3.2 Spatial resolution of ESTASI and IEF *124*
3.3 ESTASI-MS detection of a mixture of peptides separated by gel IEF *127*
3.4 ESTASI-MS in the analysis of protein mixtures separated by gel IEF *138*
4. Conclusions 140
References 141

Chapter VI. Electrostatic Spray Ionization Mass Spectrometry Sniffing for perfume fingerprinting 143
1. Introduction 144
2. Materials and methods 146
2.1 Materials *146*
2.2 Paper electrostatic-spray ionization *146*
2.3 Mass spectrometer *147*
3. Results and Discussion 147
3.1 Paper electrostatic spray ionization mass spectrometry *147*
3.2 Paper ESTASI for the analysis of a model perfume *149*

3.3 Paper ESTASI for the analysis of six authentic fragrances *151*
4. Conclusions 156
Appendix 6.0 ..... *157*
References 158

Chapter VII. Standard addition strip for quantitative electrostatic spray ionization mass spectrometry analysis: Determination of caffeine in drinks.... 160
1. Introduction 161
2. Materials and methods 163
2.1 Chemicals & Materials *163*
2.2 Sample Preparation *163*
2.3 High performance liquid chromatography quantitation of caffeine *163*
2.4 ESTASI-MS *164*
2.5 Standardization on ESTASI-MS *165*
2.6 Standard addition calibration by ESTASI-MS *165*
2.7 Standard addition strip-ESTASI-MS *165*
3. Results and discussion 167
3.1 Quantitative analysis from droplets of standard solution by ESTASI-MS *167*
3.2 Standard addition calibration of caffeine in various drinks by ESTASI-MS *168*
3.3 Method of standard addition using strip-ESTASI-MS *172*
3.4 Quantification of caffeine in saliva by strip-ESTASI-MS *177*
4. Conclusions 180
Appendix 7.0 ..... *181*
References 184

Chapter VIII. Direct analysis of desalted samples from RP-C_{18} ZipTip® by electrostatic spray ionization mass spectrometry 186
1. Introduction 187
2. Materials and methods 189
2.1 Chemicals & Materials *189*
2.2 ESTASI-MS *189*
3. Results and discussion 191
3.1 Electrostatic spray ionisation of peptide or protein from micropipette tip 191
3.2 Electrostatic spray ionisation of peptide or protein from RP-C_{18} ZipTip 192
4. Conclusions 197
Appendix 8.0 ..... *198*
References 199

Chapter IX. Summary and perspectives 201
1. Summary 202
2. Electro-elution device for protein/peptide purification using ultra narrow pH gradient gels 204
3. Two-dimensional gel electrophoresis coupled to ESTASI-MS 206

CV of Elena Tobolkina 209

Chapter I.

Introduction

1. Proteomics

1.1. Proteome and genome

From 1995, we have witnessed a renaissance in the field of proteomics and since then over 64,000 related articles have been published, indicating the breadth of scientific interest in this field. What is proteomics and where does this interest stem from? Marc Wilkins is often credited to have introduced the term "proteome", which is derived from two words PROTEins and genOME to highlight that the code for protein synthesis lies within the genes. The human genome contains up to 40,000 genes, while the corresponding proteome (*i.e.* "all proteins expressed by a genome, cell or tissue at a given time") in the human body[1] is much more complex and contains around 2 million different proteins[2]. The proteins can be found over a large dynamic range between low and high abundance (1–10^5 or 10^6) and even larger in plasma (up to 10^9–10^{10})[3]. This is further complicated by the fact that proteins are large molecules composed of one or more long chains of amino acids, existing in a variety of different stereo configurations that could potentially carry any chemical group at the side chain; more than 500 amino acids have been classified so far, 240 of them occur in nature[4]. The genome is determined by the sequence of nucleotides, but the proteome cannot be limited to only the amino acid sequences of proteins, since the proteome requires knowledge of different protein structures, physicochemical properties, which depends on the sum of amino acids masses and its position in the polypeptide chain[5, 6]. The size, shape and functions of proteins vary after the post-translational modifications (PTMs) of some functional groups[7]. Several types of post-translational modifications are known, such as phosphorylation (addition of a phosphate group to the residue of one or several amino acids)[8], glycosylation (neutralization of negative charges)[9], acetylation (positive charge suppression)[10] *etc.*

In general, the field of proteomics can be divided into three parts: *profiling*, which relates to identification and quantitation of proteins from biological samples, *structural* – where tertiary protein structure is described and *functional proteomics* where the protein functions are identified through protein-ligand interactions and PTM studies. Since it is very difficult to predict and determine all the components of a protein complex, its function based on homology or its three-dimensional structure stays one area of "greatest promise"[11]. The aim of proteomics is to identify and

characterize all the proteins expressed by an organism or a tissue, as well as investigating biomarkers related to specific diseases. The purpose of biomarker search is to find a particular protein, that can be used as the target protein in disease diagnosis[12].

1.2. Strategies in proteome research

The qualitative and quantitative analysis of complex protein mixtures has become one of the main topics in many laboratories worldwide. Several strategies have been employed for protein analysis in proteomic studies, including bottom-up, top-down and more recently appeared middle-down proteomics (see Figure 1.1).

"Bottom-up" proteomics is a method of protein identification by characterization of amino acid sequences through proteolytic digestion using mass spectrometry. When a bottom-up analysis is performed on a mixture of proteins, it is called shotgun proteomics[13, 14], where the biological sample is, firstly, digested by proteolytic enzyme (*e.g.* trypsin) that cleaves peptide chain of proteins at well-defined sites (lysine or arginine) to create peptide mixtures. The generated mixture is separated by liquid chromatography (LC) and characterized by tandem mass spectrometry (LC-MS/MS)[15]. Using the collision induced dissociation technique (CID), *b, y*-fragments are generated and the peptide sequence is obtained by comparing tandem mass spectra with the theoretical pattern derived from peptide sequences using a protein database. When electron capture dissociation (ECD) or electron transfer dissociation (ETD) is used, *c, z*-fragments are generated while the PTMs, *e.g.* phosphate groups, can be retained bringing advantages in PTMs studies[16]. The main advantage of this strategy is to achieve high-throughput analyses. However, there is a couple of very important limitations: (i) limited protein sequence coverage due to the fact that only several peptides are identified during the analysis, (ii) the tryptic peptides size is relatively small (~8–25 residues long) that leads to sample complexity and limitations in sequence coverage, as well as loss of information about PTMs, which can carry the information about structural and functional activities of proteins[17].

When the large peptide fragments in the 5-15 kDa range are produced by the cleavage of rarely expressed amino acids or combinations of amino acid residues such approach is called "middle-down" proteomics[18]. The digestion can be done using the

enzyme that cuts less frequently than trypsin[19, 20], or by chemical cleavage or method like microwave-assisted acid hydrolysis[21]. Studies of such large peptide fragments may give advantages, such as information about PTMs and high sequence coverage, due to the fact that relatively short peptide chain can be easily separated and ionized compare to the intact protein analysis.

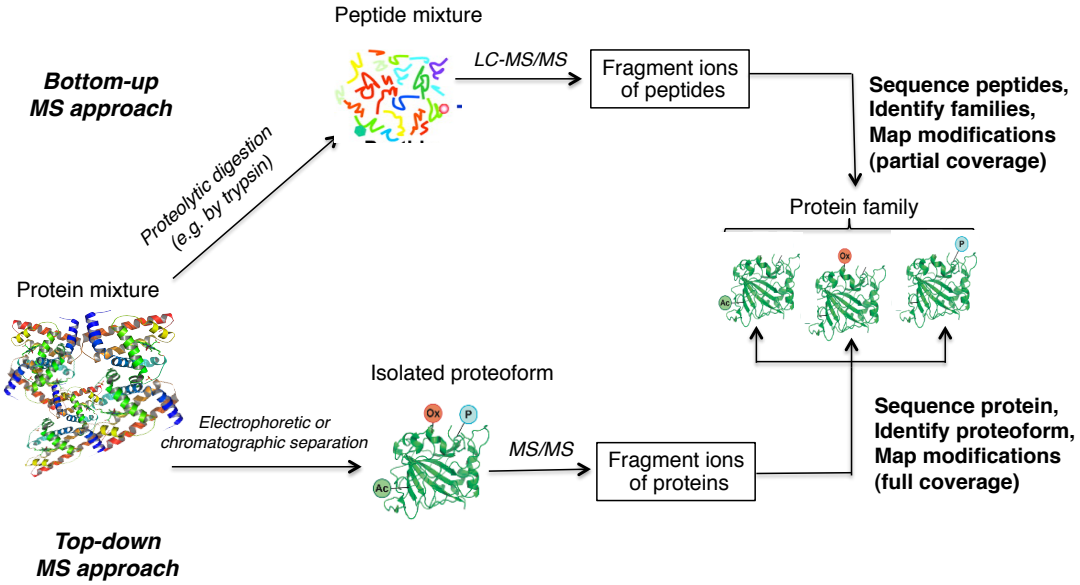

Figure 1.1. Schematic representation of bottom-up and top-down proteomics.

Another strategy called "top-down" proteomics uses mass spectrometric analysis in order to characterize intact proteins from complex biological mixtures without any digestion to peptides. This approach can provide identification of proteoforms, *i.e* a protein form with specific genetic or chemical modifications. Analysis by two-dimensional (2D) gel electrophoresis is one of the examples of top-down proteomics; however, to increase the efficiency and to get more structural information about the protein, as well as ability to detect the combinations of PTMs can be achieved by mass spectrometry[22]. ECD and ETD used in top-down approach were shown to produce higher sequence coverage then CID[23]. Nevertheless, the top-down method is used less compared to the bottom-up strategy due to difficulties in protein ionization, fragmentation in the gas phase, very high cost of high-resolution mass spectrometers (*e.g.* FTICR), and in-general low throughput.

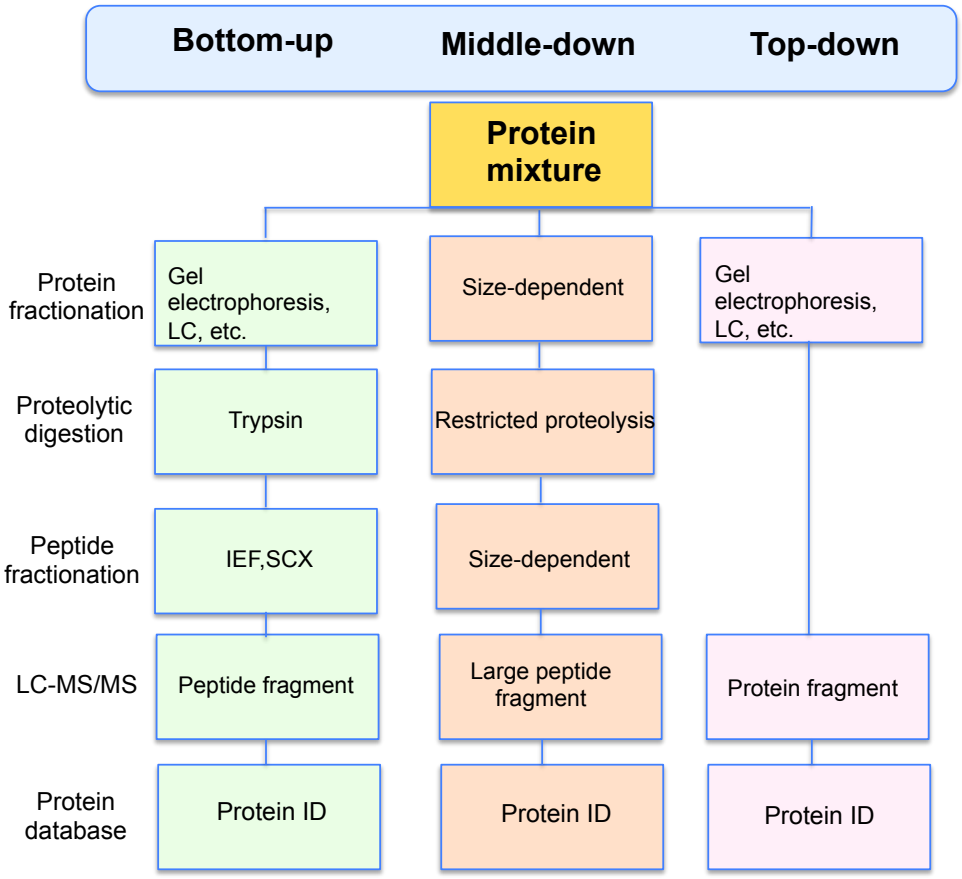

Figure 1.2. Three proteomic strategies: bottom-up, middle-down and top-down, adapted from Zhang *et al*., Chemical Reviews, 113, 2343,2013[24].

To date, the most commonly used methodology is shotgun proteomics, which became the method of choice for a wide variety of applications, from discovery of biomarkers for different diseases to quality control in the cosmetics, food, and pharmaceutical industries[25, 26].

1.3. Techniques in proteome research

The fact that there is not a single method available that can identify and quantify a complete set of proteins at once, due to their complexity, the combination of highly sensitive separation methods with mass spectrometry (MS) identification is a requirement in most proteomic studies[27].

High-resolution two-dimensional (2D) gel electrophoresis followed by highly-sensitivity MS is one of the most powerful strategies in proteomics for the last 20 years[28, 29]. This approach is based on the separation of proteins according to their isoelectric point (p*I*) in the first dimension by isoelectric focusing (IEF) and to their molecular weight in the second dimension by sodium dodecyl sulfate polyacrylamide

gel electrophoresis (SDS-PAGE). The separation parameters are orthogonal and independent even though they occur in the same matrix[30]. After separation, the proteins can be visualized by different staining protocols (Coommasie brilliant blue or Silver staining) or cut out of the gel, digested and analyzed by MS[31], or a gel imaging system can be used for the analysis and archiving of protein spots on the polyacrylamide gel. There are several commercial gel imagers, such as Gel Doc™ XR+ system by Bio-Rad based on fast, high-resolution, and high-sensitivity analysis of samples[32].

The isoelectric point distribution of the whole proteome can be estimated by so called virtual 2D gels, by plotting p*I* of the proteins against their molecular weight. In all proteomes the 2D plot looks bimodal with low amount of fractions at p*I* around 7.0 due to proteins insolubility and low probability of constructing a protein with such p*I* by naturally occurring amino acids[33].

Electrophoretic and chromatographic techniques are the main tools of complex sample separations in proteomics. The peptide mixtures are mainly separated by different modes of HPLC (normal-phase chromatography (NP), ion-exchange chromatography (IEX), size-exclusion chromatography SCX, affinity and reverse-phase chromatography (RP)[34]) and CE or a combination of these two techniques. An advantage of using electrophoretic and chromatographic techniques is that complex protein/peptide mixtures are resolved based on their physicochemical properties.

Multidimensional separation of complex mixtures involves the use of several orthogonal and compatible methods. Wagner *et al*. have proposed the use of multidimensional HPLC system for the separation of proteins with a molecular weight less then 20kDa; around 1000 peaks have been resolved in 96 min[35].

In most HPLC and CE multidimensional approaches, the proteins are digested prior the analysis that possess a better solubility in a wide range of solvents; however, increasing the number of species in the sample. LC based separation techniques provide a good compatibility with MS and a high peak capacity. Hunt *et al.* proposed the use of immobilized metal-affinity chromatography (IMAC) followed by HPLC-MS for the identification of the whole cell lysate; using this strategy over 1000 phosphopeptides were identified[36]. Around 11000 unique phosphopeptides were detected using weak anion exchange (WAX) chromatography followed by LC-MS analyses[37]. Size-exclusion liquid chromatography (SEC) followed by reversed-phase liquid chromatography (RPLC) and monitored by matrix-assisted laser

desorption/ionization time-of-flight (MALDI-TOF) MS was used for isolation and identification of two nonnative proteins from *E.coli* extract[38].

On-line and off-line RP-HPLC-CE methods were proposed to show the efficiency of analyzed protein digests. Moore and Jorgenson used RPLC-CE separations to resolve complex peptide mixtures; the second dimension was designed to be faster than the first one, allowing the analysis of many eluted samples[39, 40]. Issaq *et al.* used offline RP-HPLC-CE for the separation of protein digest, where the fractions from the LC column were collected every 30 s and examined by capillary electrophoresis (CE) allowing the experiment to be completed in 1h[41].

Besides 2D electrophoresis, OFFGEL and IEF followed by LC were employed for the separation of protein mixtures. Xiao et *al.* used ampholyte-free IEF device for fractionation digested human serum sample. Collected fractions were further analyzed by microcapillary μRPLC-MS/MS; 844 peptides (437 proteins) were detected[42]. Two-stage OFFGEL electrophoresis followed by proteolytic digestion of intact proteins and its analysis by LC-ESI-MS/MS were able to identify 660 peptides (81 proteins) from human plasma[43, 44]. About 1373 proteins were detected by coupling OFFGEL electrophoresis to nano-LC-MS/MS detection[45]. The 2D-IEF–RPLC system was also suggested as a tool for cellular protein separation[46]. Gel-enhanced LC-MS (GeLCMS) approach was designed to improve the separation resolution; a protein mixture is separated by gel electrophoresis followed by in-gel digestion of the bands, and, subsequently, with further analysis of peptides by LC-MS/MS[47]. The membrane proteins from a human colorectal carcinoma cell were separated by SDS-PAGE, the bands were cut out from the gel, digested and analyzed by RP-HPLC followed by ESI-MS analyses, over 284 proteins (including 92 membrane proteins) were identified[48].

2. Gel-based separation approaches

In 1937, Tiselius published a seminal work, in which a new apparatus for moving boundary method, for studying the electrophoresis of proteins was introduced[49]. The proposed device consists of a U-shaped cell with a buffer solution and electrodes loaded at each end. The sample applied could be any mixture of charged compounds. After applying an electric field, ions were migrated to either the anode or cathode depending on their charges[50]. Since this pioneering work, different forms of

electrophoresis have been employed with the two main parameters used for proteins separation, such as electrophoretic mobility and the resistance of medium. To resolve a complex mixture by 1-D electrophoresis was a challenging task, until 1956, when Smities and Poulik proposed the combination of two electrophoretic processes, where "More than fifteen resolved components could be seen". The first dimension was carried out on a filter paper separating the serum proteins by free-solution mobility and by molecular weight in a starch gel in the second dimension[51]. Ashton used agar in the first dimension and starch gel in the second dimension to separate serum proteins[52].

In 1962, Raymond and Aurell employed two acrylamide gels of different concentrations for serum proteins separation[53]. Since 1964, the combination of acrylamide gels named as "orthogonal gel electrophoresis" has been used till now in two-dimensional gel electrophoresis[54]. Presently, two-dimensional gel electrophoresis remains as one of the most important high-throughput tools for proteome research.

2.1. Isoelectric focusing (IEF)

IEF is a high-resolution electrophoretic technique for separating amphoteric species according to their isoelectric points (p*I*) in a stable pH gradient media and under the application of an electric field[55]. The p*I* of a protein is the pH, at which both the net charge and hence the electrophoretic mobility of the protein are equal to zero (see Figure 1.3).

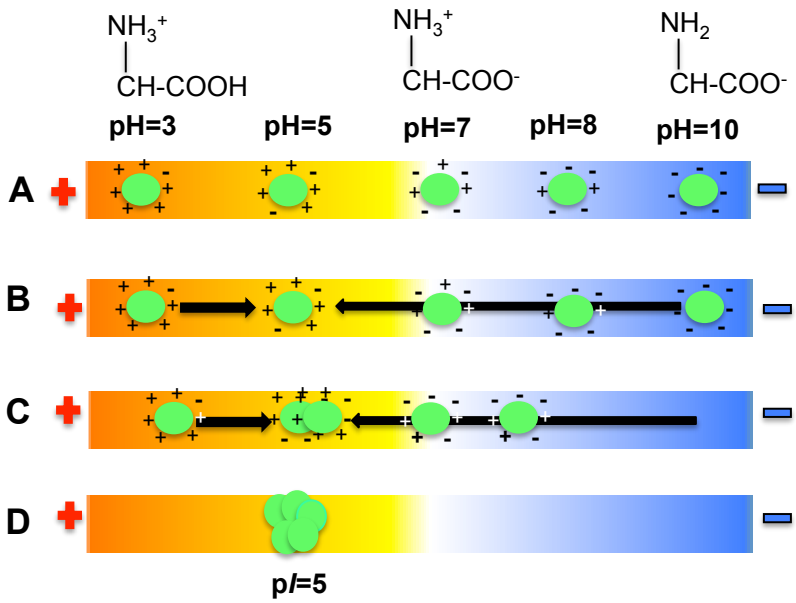

Figure 1.3. Schematic representation of the isoelectric focusing technique. The sample is placed in the gel media where the electric field is applied. Depending on their charge the molecules start to migrate inside the gel until reaching their isoelectric point. Adapted from Koshel *et al.*, Proteomics, 2012[56].

The samples fractioned by IEF are mainly peptides and proteins; however, other entities, such as bacteria cells or amphoteric nanoparticles, can be also separated by IEF[57]. When a mixture of proteins or peptides is applied in a stable pH gradient medium, a net charge specific for each protein will be generated depending on the pH of the gel, its functional groups capable of electrolytic dissociation, and the interaction with other molecules or ions. In other words, the net charge of a protein at a given pH is the sum of all the charges coming from the present ionizable groups in the protein, and can be calculated in a first approximation by the equation[58]:

$$z(\mathrm{pH}) = -\sum_{i \in A^-} \frac{1}{1 + \frac{10^{-\mathrm{pH}}}{K_i}} + \sum_{i \in A^+} \frac{1}{1 + \frac{K_i}{10^{-\mathrm{pH}}}} \tag{1.1}$$

where K_i is the ionization constant of the ionizable group of the corresponding amino acid, while A^- and A^+ denote the negative and positive charge of amino acids. The positive charge can be provided by amino acid residues of histidine (H), arginine (R), lysine (K) and the N-terminus. The negative charges are given by amino acid residues of aspartic acid (D), cysteine (C), tyrosine (Y), glutamic acid (E) and the C-terminus.

Therefore, the protein's net charge and its corresponding p*I* depend strongly on the basicity or acidity strength of the present amino acid residues (*e.g.* acidity or

basicity dissociation constant, *vide infra*) and their interactions with another amino acids inside the protein sequence.

When a protein is placed in a pH gradient medium, in the presence of an electric field, the charged proteins will be forced to migrate to the electrode of opposite charge across the pH gradient. The net charge of the protein will change during its migration due to the pH gradient. When reaching a medium with a pH equal to its p*I*, the protein will no longer migrate under the electric field, but diffuse away from its p*I* zone because of concentration gradients until a net charge is generated again causing the protein to move back. Thus, the concept of isoelectric focusing can be described as:

$$J_i = -c_i \tilde{u}_i \operatorname{grad} \tilde{\mu}_i = - c_i \tilde{u}_i \operatorname{grad} \mu_i - c_i \tilde{u}_i z_i F \operatorname{grad} \phi \qquad (1.2)$$

where J_i is the flux of a species, ϕ is the electrical potential, c_i is the concentration, μ_i and $\tilde{\mu}_i$ are chemical and electrochemical potential of the analyte, z_i corresponds to the analyte charge and $\tilde{u}_i$ is the electrochemical mobility.

If the electric field is applied over a long enough period to focus all the proteins close to their respective p*I*'s, a steady state separation of proteins can be achieved and the conservation law can be written as[59]:

$$\frac{\partial c_i}{\partial t} = -\operatorname{div} J_i \ = 0 \qquad (1.3)$$

In the case when the concentration and the charge of analyte is changing across the pH media, the equation become

$$-\frac{\partial}{\partial x}\left[-\frac{c_i \tilde{u}_i RT}{c_i}\frac{\partial c_i}{\partial x_i} - c_i \tilde{u}_i z_i F \frac{\partial \phi}{\partial x}\right] = 0 \qquad (1.4)$$

Since the charge of an analyte at its p*I* is zero, the concentration is maximum. Equation (1.4) can be rewritten as equation (1.5), meaning that the concentration gradient is proportional to the electric field.

$$RT \frac{\partial c_i}{\partial x} = \ c_i \ z_i FE \qquad (1.5)$$

In 1961, Svensson (later called Rilbe) proposed the idea to establish a stable pH gradient in the gel through the use of amphoteric species (*e.g.* carrier ampholytes)[60]. According to Svensson, a highly reproducible IEF electrophoresis

requires not only a stable pH gradient, but also a good electrical conductivity along with a high buffer capacity[60]. These requirements have been achieved after the work of Vesterberg, who synthesized the ampholytes that constitute the mixture of aliphatic oligo-amino and oligo-carboxylic acids[61, 62]. After introducing the carrier ampholytes it was possible to separate two myoglobins based on a p*I* of only 0.02 pH[63].

The buffer capacity of carrier ampholytes near their isoelectric points is an important parameter, because they should exhibit a stronger buffer action than the proteins to control the pH gradient of the gel. The buffer capacity, β, is defined as any change in pH caused by the addition of acid or base to a solution. β can be written as:

$$\beta = \frac{\mathrm{d}c_{\mathrm{B}}}{\mathrm{d(pH)}} \tag{1.6}$$

where c_{B} is the concentration of a base or an acid. The higher the buffer capacity of an ampholyte, the lower the changes in pH, which can be affected by the addition of acid or base. Rilbe[64] has demonstrated that the maximum molar buffer capacity, β_i, of an ampholyte is attained when

$$\Delta \mathrm{p}K = \mathrm{p}K_2 - \mathrm{p}K_1 \geq \log 4 \tag{1.7}$$

From equation (1.7), it can be seen that, β_i is decreased when increasing $\mathrm{p}K_2 - \mathrm{p}K_1$ across the p*I*. Therefore, ampholytes with $\Delta \mathrm{p}K$ greater than 4 have low buffering capacity close to their p*I* and they should not be employed.

In order to cast a linear stable pH gradient, a mixture of ampholytes that present a wide range of p*I*'s, which will form the pH range of interest should be added to the gel. Similar to IEF of proteins, the focusing of an ampholyte around its p*I* can be obtained as a result of the balance between the natural diffusion process and the imposed electrophoretic movement. As the carrier ampholytes diffuse faster than proteins, a steady state will be reached very quickly and a pH gradient across the gel is established[46]. This procedure can be expressed by[55]:

$$c_i u_i E = D \frac{\mathrm{d}c_i}{\mathrm{d}x} \tag{1.8}$$

where c_i, u_i, E and D are the concentration of the ampholyte, its electric mobility ($u = zF\tilde{u}$, where $\tilde{u}$ is the electrochemical mobility), the electric field strength and the ampholyte diffusion coefficient, respectively. The left side of equation (1.8) represents the migration flux of the ampholyte, while the right side describes the diffusion flux of the ampholyte.

This differential equation determines the final steady state obtained in the IEF separation, in which the ampholytes are concentrated in their respective area of p*I*. Additionally, it can be assumed that the change in pH and electrical mobility is constant at the vicinity of its p*I*:

$$p_i = -\frac{\mathrm{d}u_i}{\mathrm{dx}} = -\left(\frac{\mathrm{d}u_i}{\mathrm{dpH}}\right)\left(\frac{\mathrm{dpH}}{\mathrm{dx}}\right) \tag{1.9}$$

where p_i is a positive value that represents the first approximation linear variation of the electric mobility as a function of the distance x. The variation in the charge as a function of the pH around the corresponding p*I* value can be written as the linear relationship:

$$p_i = -u_i/x \tag{1.10}$$

If one were to substitute equation (1.10) into equation (1.8), then equation 11 would be obtained:

$$-\frac{p_i E}{D}\mathrm{xdx} = \frac{dc_i}{c_i} \tag{1.11}$$

Integrating the last equation gives a Gaussian distribution of ampholyte in the region near the isoelectric point of the ampholyte in which the maximum concentration of c_0 can be achieved.

$$c_i = c_i^{max}\exp\left(-\frac{Ep_i x^2}{2D}\right) \tag{1.12}$$

According to this equation the quality of the isoelectric focusing of an ampholyte will depend on the strength of the electric field, and the diffusion coefficient of the species. The standard deviation (σ) of this Gaussian distribution is:

$$\sigma = \frac{D}{Ep_i} \tag{1.13}$$

Then the resolution R between two ampholytes can be defined as[65]:

$$R = \frac{\Delta \mathrm{p}I}{\overline{\Delta\sigma}} = \frac{2\Delta \mathrm{p}I}{\sigma_1 + \sigma_1} \tag{1.14}$$

With equations (1.9), (1.13) and (1.14), it is seen that resolution in IEF can be improved by the use of a strong electric field. The resolution can be increased by separation of ampholytes with small diffusion coefficients and sharp variations of d*u*/dpH at their p*I* zones.

Despite the enormous success of IEF based on carrier ampholytes, the technique presents certain drawbacks. For instance, due to the low molecular weight

of carrier ampholytes, they have a high rate of diffusion in the gel and might diffuse away from its p*I*. If a long focusing time is needed, conductivity gaps can occur and the proteins will migrate out of the gel[66]. Another disadvantage is that the carrier ampholytes have to be washed out from the gel before staining. To overcome these limitations, an alternative strategy for creating stable pH gradients without carrier ampholytes has been explored. At the end of 1980s Gasparic *et al.* patented a new set of buffering monomers, covalently bond to the gel matrix, which provides a stable pH gradient, named Immobilines[67]. Almost any pH gradient (narrow or wide pH) can be achieved as they are bound to the structure of the polyacrylamide gel, making possible the separation of very acidic or basic proteins. Since the pH gradient is completely immobilized and stable, reproducible linear gradients can resolve proteins with p*I* differences of 0.01 pH units. Immobilines buffers are widely used in the immobilized pH gradient (IPG) gels[68].

Besides the instruments that use IPG gels, like Hoefer's IsoPrime[68], as well as "ampholyte-free" Optifocus and Rotolyte buffer systems and free-flow techniques that use a thermal gradient to produce a stable pH gradient to separate charged species[69], alternative methods to IEF have been developed. The first alternative instrument called counteracting chromatographic electrophoresis (CACE) was proposed by O'Farrell, based on the difference between the velocity of a protein in a flow field and its electrophoretic velocity in an opposing electric field to separate components of a mixture[70]. Recycle zone electrophoresis (RZE) introduced by Ivory uses buffers that penetrate through the membrane to altered the crossflow so the proteins whose electrophoretic mobilities suit the "focusing window" would be collected near the feed loop, the rest would migrate out of it[71]. Later, Koegel and Ivory introduced an electric field gradient focusing (EFGF), where the proteins are focused in a band according to their electrophoretic mobilities by applying a gradient of electric field along the length of a separation column[72]. Another alternative technique, called dynamic field gradient focusing (DFGF), separates charged species according to their electrophoretic mobilities by controlling the electric field gradient[73].

2.1.1. Immobilized pH gradient (IPG) gel

The IPG principle was firstly introduced in a patent of Rosengren *et al.* which describes an acrylamide gel matrix that is co-polymerized with a specific mixture of amphoteric monomers (see Figure 1.4)[74]**.** These monomers are weak acids or bases with known p*K* values when mixed together with acrylamide, bisacrylamide, tetramethylethylenediamine (TEMED), and ammonium persulfate (APS) for preparation of a polyacrylamide gel. The general structure of the monomers is CH_2=CH-CONH-R, where R contains either carboxyl or tertiary amino group. The immobilized pH gradient is obtained by mixing both acidic and basic monomers in the range of pH 3 to 10 in different ratios. The pH of acidic or basic Immobilines is given by Henderson-Hasselbach equation:

$$\mathrm{pH} = \mathrm{p}K_{\mathrm{A}} + \log\left(\frac{c_{\mathrm{A}}}{c_{\mathrm{A}} - c_{\mathrm{B}}}\right) \tag{1.15}$$

In the case of a basic Immobiline:

$$\mathrm{pH} = \mathrm{p}K_{\mathrm{B}} + \log\left(\frac{c_{\mathrm{B}}}{c_{\mathrm{B}} - c_{\mathrm{A}}}\right) \tag{1.16}$$

where c_{A} is the molarity of the acidic immobiline with p*K*=pK_{A} and c_{B} is the molarity of the basic Immobiline with p*K*=pK_{B}.

For casting linear and no-linear gradients in the gels, Bjellqvist graphics can be used[68, 74]. The buffering capacity of IPG gels must be high enough (5-6 mequiv·l^{-1}·pH^{-1}) to focus analytes in a band[75].

Another important aspect is the pore size of the gel that might or might not limit the migration/diffusion of molecules within the gel. Typically, the total concentration of acrylamide present (designated as % T) and the degree of cross-linker (N,N-methylenebisacrylamide) (% C) are the parameters that determine the pore size of the gel. Generally, as the total amount of acrylamide increases, the pore size decreases. For separation of proteins in a range of 15-70kDa a stock solution of 40 or 30% T with 2.5 or 3.3% C are commonly used. 10%T is useful to resolve the proteins with molecular weight of 150 kDa. The preparation of gels with less then 7%T should be avoided, as they are very fragile[76].

$$\mathrm{T} = \frac{(a+b)\times 100}{\mathrm{V}}[\%] \tag{1.17}$$

$$\mathrm{C} = \frac{b\times 100}{(a+b)}[\%] \tag{1.18}$$

where *a* and *b* are the mass of acrylamide and methylene bisacrylamide in grams, V is the volume of the solution in mL[77].

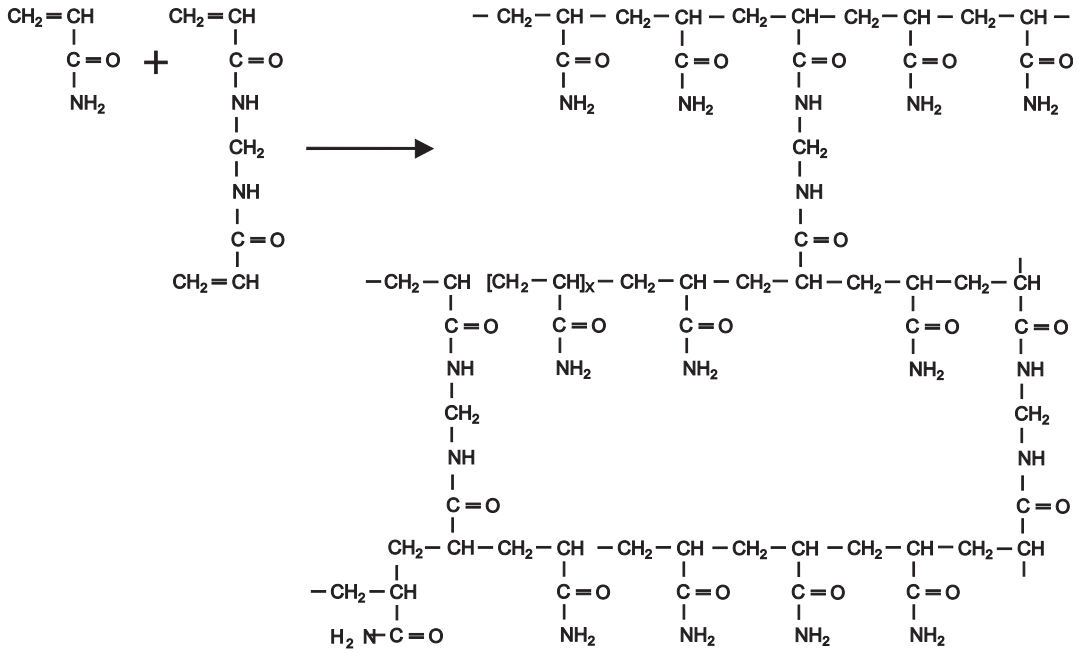

Figure 1.4. Polymerization reaction of acrylamide and methylene bisacrylamide performed to achieve polyacrylamide gel. The reaction takes place in the presence of persulfate radicals and TEMED, which plays the role of catalyst. Reprinted from S. Magdeldin, Gel electrophoresis principles and basics. InTech, Croatia, 2012[78].

IPG gels are commonly used in IEF electrophoresis and have been commercialized by companies such as GE Healthcare and Bio-Rad. IPG gels are casted on plastic (polyester) films to improve their mechanical stability and to make their manipulation easier. IPG gels facilitate the use of additives, such as urea and detergents, for optimal performance. Thanks to the very good reproducibility and high protein loading capacity (up to 100 mg/mL), no drift of pH gradient is observed during electrophoresis, and direct staining is possible; however, the use of IPG gels suffers from several limitations. First, the gel rehydration can take a couple of hours before the gel is ready for use. IPG have to be equilibrated in order to maintain protein solubility before being loaded on top of the SDS-PAGE and to reduce disulfide bonds. Membrane proteins do not migrate into the second dimension once they are stacked in the IEF, basic proteins are hard to be resolved in that case non-equilibrium pH gradient electrophoresis (NEPHGE) technique can become the method of choice[79]. IPG and NEPHGE methods were compared between each other

and it was shown that IPG 3-10 is reliable for the analysis of acidic proteins, while NEPHGE can be used in all the pH ranges and is efficient for the separation of basic proteins[80].

2.1.2. OFFGEL electrophoresis (OGE)

OFFGEL electrophoresis is a concept developed several years ago in our laboratory[81] and commercialized by Agilent Technologies in 2006[82]. It separates proteins or peptides according to their isoelectric points, while the separated components are recovered in liquid fractions. The principle of this technique is as follows: a frame that contains several chambers (*i.e.* the same dimensions) of the same size is located on top of an hydrated IPG gel in such a way that the gel is divided between the number of chambers, while at the same time allowing the connection between chambers through the gel. Afterwards, a mixture of buffer and sample solution (≈150 μl) is loaded on top of the gel and an electric field between the extremes of the gel is applied, resulting in the movement of proteins from one chamber to another, according to their p*I*'s (Figure 1.5). After the separation, only the neutral species (pH= p*I*) stay in solution.

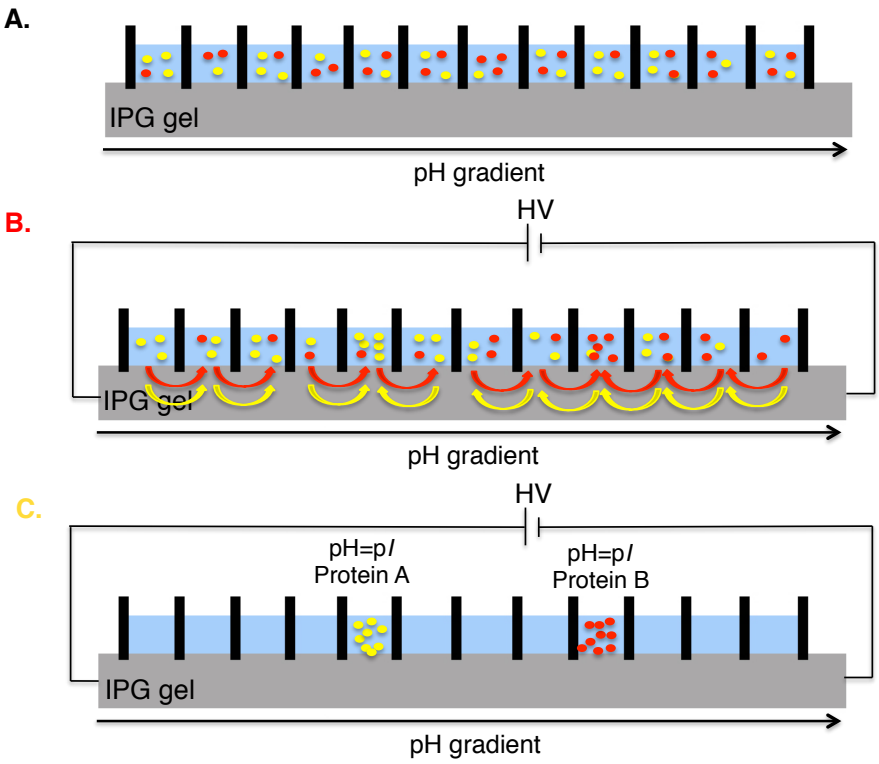

Figure 1.5. Schematic representation of OFFGEL electrophoresis principle. **A.** Firstly, the proteins samples diluted in a buffer are placed in the wells. **B.** An electric field is applied, and the proteins start migrating to their isoelectric points. **C**. When the proteins reach their isoelectric point (p*I*=pH) they can be recovered in liquid solution.

OFFGEL technique can be performed in a multi-well format and the resolution of 0.1pH unit for protein separation can be achieved[83]. In general, the resolution of the separation depends on the length of the IPG strip, the number of wells, as well as the pH range of the gel, which is in contact with analyte solution. By increasing the number of wells from the standard 24-well to 48-well frame it is possible to double the separation resolution of proteins with a small p*I* difference and PTMs (*e.g* phosphorylations)[84].

The separation recovery depends on the height of the well (h_{well}) and the height of gel (h_{gel}), the theoretical recovery of 96%, 82% and 50% were obtained for the ratios h_{well}/h_{gel}=10, 4, 1, respectively[58]. However, the theoretical values are far from the reality, since the h_{well} is not fixed and the liquid amount can change during the electrophoresis. However, the sample recovery can be improved more than 30% by adding the extraction step (50% acetonitrile/0.1% formic acid for only 15 min) directly after OFFGEL separation[85].

OFFGEL electrophoresis provides: (i) protein/peptide recovery from the liquid phase allowing direct analysis of the fractions by other analytical techniques, such as mass spectrometry or HPLC; (ii) physicochemical information of the sample, such as the p*I* value; (iii) high resolution of separation, high sample loading, control of a well-defined pH range, and flexibility in choosing the desired pH gradient[86]. Hörth *et al.* reported the first application on OGE where up to 90 % of the peptides from *Escherichia coli* extract were identified from 1 or 2 samples showing high resolving power of the technique[87]. Though, OGE suffers from a couple of limitations, where the main disadvantage is the long separation time from several hours to 2-4 days. The presence of salts in a sample can also influence the fractionation time. It was reported by García-Otero *et al.* that the high concentration of salts in a sample could lead to problems in adjusting the electric current making the OFFGEL initialization impossible[88].

OFFGEL electrophoresis has been used in a wide variety of applications, such as fractionation of platinum-binding proteins[89], human plasma proteins[90, 91], post-mortem cerebrospinal fluid[92], and cancer diagnosis[93]. It has been demonstrated to be a useful tool in proteome analysis.

2.2. SDS-PAGE electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate proteins according to their molecular weight based on their migration under the influence of applied electric field. This is achieved through protein denaturation by a detergent, namely sodium dodecyl sulfate (SDS), which is employed in order to unfold the proteins, converting them to linear molecules, by breaking the internal bonds; thereby, disrupting their original secondary and tertiary structure. SDS forms a protein-SDS complex, with the number of SDS molecules being roughly proportional to the protein's length. Therefore, all the proteins will have approximately the same mass-to-charge ratio. In addition, the coating of the protein with negatively charged SDS molecules allows the proteins to migrate in the second dimension under an electric field towards the positive (+) electrode (*i.e.* anode). In this case, protein separation is achieved owing to the proteins size, since small proteins will move faster than larger ones. As a consequence, proteins can be separated according to their size or molecular weight[94] (see Figure 1.6).

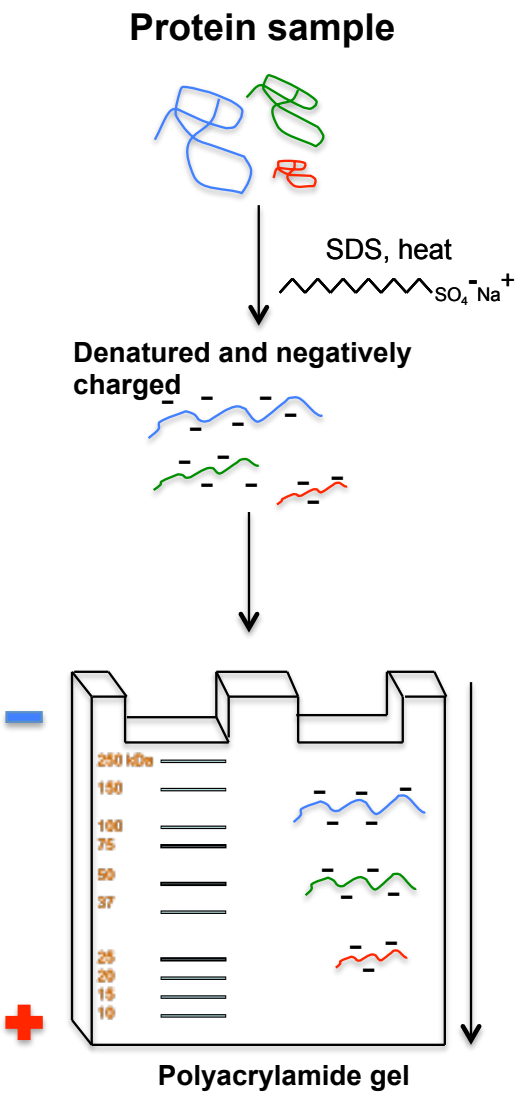

Figure 1.6. Scheme illustrating of SDS-PAGE principle. SDS is added to the protein sample in a 1.4:1 ratio as a consequence the proteins are denatured and negatively charged. The sample is loaded on the gel and an electric field is applied causing the proteins to migrate with a velocity that is proportional to the logarithm of their molecular mass.

SDS-PAGE in contrast to IEF is not a steady-state technique; therefore, special attention has to be paid in order to avoid the proteins migrating out of the gel. For this reason, a marker that migrates faster than the proteins is employed to determine the end of separation. After separation, the gel should be fixed by acidic (acetic acid) or alcoholic solution (ethanol, methanol) depending on the chosen dyeing protocol. Most commonly Coomassie Brilliant Blue staining or silver staining are used to visualize protein spots in a gel. The detection sensitivity of 100 ng/spot can be achieved by using Coomassie brilliant blue staining, while silver staining is even more sensitive- up to 1 ng/spot[95].

2.3. Two-dimensional (2D) gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was introduced by O'Farrell[96] and Klose[97] through the combination of IEF and SDS-PAGE. Anderson and Anderson used 2D-PAGE for analysis of human plasma proteins and were able to resolve 300 proteins spots[98]. In 2D electrophoresis, proteins are separated according to their p*I* in the first dimension on the IPG strips and by molecular weight (Mw) in the second dimension using SDS-PAGE; this provides a unique capacity for the resolution of complex mixtures of proteins and affords the analysis of hundreds or even thousands of spots (see Figure 1.7). Protein separation can be achieved as low as 0.1 p*I* unit and 1 kDa in MW[99].

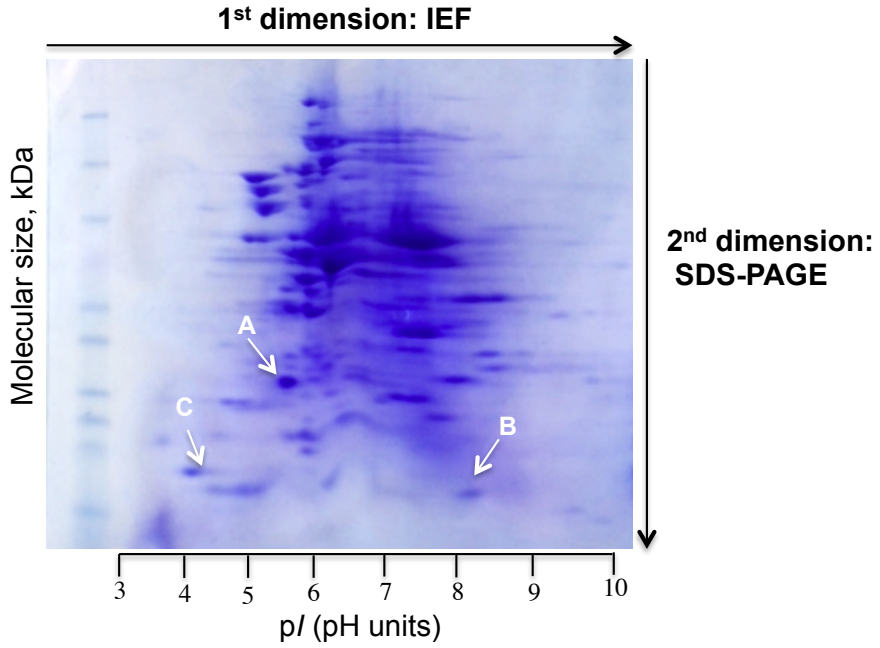

Figure 1.7. 2D-PAGE scheme with the first (IEF) and the second (SDS-PAGE) dimension, where A, B, C are the spots of the proteins.

There are several ways to run electrophoresis by applying either constant voltage or constant current. In the case of applying a constant voltage, the resistance increases, while the current decreases resulting in a longer separation time. While the constant current applied allows faster separation, but produce more heat. Additionally, the pore size of a polyacrylamide gel can be adjusted by changing the concentration of acrylamide and the cross-linking reagent in order to introduce sieving features to the gel[100]. The second dimension can be performed in horizontal or vertical systems[101].

Two-dimensional electrophoresis is an established technique considered to be the best option for high-resolution profiling of low abundance proteins and is currently the method of choice for the quantitative comparison towards changes in protein profiles of cells, tissues, or across an entire organism[102, 103]. 2D-PAGE is commonly used in various fields of biological and biomedical science for studying complex biological systems, the chemical effects on human and animals, mutation, and various diseases[104]. Hoving *et al.* used the IPG strips, employing a very narrow pH range in the first dimension of 2D-PAGE to separate the proteins from within the B-lymphoma cell; in this way, 1500 spots were detected from a single gel and about 5000 spots were detected after using six ultra-zooms gels[105]. Wildgruber *et al.* have found that a much higher amount of proteins (2285 compared to 755) can be obtained by using the narrow IPG strips instead of the gels with a pH from 3-10[106]. Using this powerful technique Vuong *et al.* demonstrated that it was possible to separate around 10,000 individual abundant proteins per cell at a time[107, 108]; however, around 76% of the proteins could not be detected due to their low concentration, which is lower than standard staining methods.

2D electrophoresis can resolve thousands of proteins and it is a very economical technique, since no special equipment is required. Its main limitations are related to the protein detection sensitivity; the experimental time and the co-migration of proteins that introduces artifacts and that make the visualization of protein spots difficult[109]. Proteins with MW higher than 250 kDa and less then 10 kDa cannot be resolved in polyacrylamide gel; however, they can be separated in agarose gel[110]. Also, more abundant proteins can mask low abundance proteins. To avoid this problem, affinity chromatography can be the method of choice[111]. Moreover, proteins with extreme p*I* values (*i.e.* below 3 and above 10) or extreme molecular weight might be dismissed during the separation due to the limitations of the commercially available strips. For this reason, strips with the pH ranges up to 12 have appeared on the market[112]. Hydrophobic and membrane proteins are poorly represented due to solubility problems occurring during transfer from one gel to another. This problem can be partly solved by prefractionation the proteins with Triton-X114, chloroform/methanol or sodium carbonate washes, as proposed by Santoni *et al.* [113].

3. Mass spectrometry

Two soft ionization methods for large biomolecules were introduced in the late 80s namely matrix-assisted laser desorption ionization (MALDI)[114] and electrospray ionization (ESI) for which Fenn and Tanaka received the Nobel Prize in Chemistry in 2002[115, 116]. In 1993, mass spectrometry has rapidly become a "major milestone" for proteomics application after the work of Henzen *et al.* where the proteins were identified after 2D electrophoresis[117].

The ability to provide highly accurate molecular weight on intact molecules makes MS unique. Mass spectrometry (MS) is a two-step method: firstly, the analyte is volatilized and ionized in the ion source of mass spectrometer and afterwards the measurement of the mass-to-charge ratio (m/z) is taken place in the "heart" of the instrument-mass analyzer, that includes ion trap (IT), Orbitrap, ion cyclotron resonance (ICR), quadrupole (Q) or time-of-light (TOF)[118]. The combination of ESI or MALDI with different analyzers provides different types of mass spectrometry techniques. All analyzers have their own specific properties in mass resolution, speed, accuracy, dynamic range and possibility to perform tandem MS. This has prompted the development of complex multistage instruments, such as hybrid quadrupole time-of-light (Q-Q-TOF) and tandem TOF-TOF instruments[119].

3.1. Matrix-assisted laser desorption ionization

In 1988, F. Hillenkamp, M. Karas and co-workers have developed matrix-assisted laser desorption ionization (MALDI)[114]. The principle of MALDI is to deposit the sample of interest on a conductive plate and mix it with a matrix in a ratio of 1:1000[120]. The ions are irradiated with laser pulses and accelerated to a mass detector by the application of an electric field between the plate and detector (see Figure 1.8)[121]. The ionization mechanism is still not completely elucidated. Three different pathways are proposed: (1) photochemical ionization process, in which ions are produced by protonation or deprotonation between sample and matrix in the gas phase[122, 123]; (2) cluster ionization approach, in which it is considered that laser adsorption cause ion desorption by desolvation of matrix molecules[124, 125]; and (3) pseudo-proton transfer model, where it is considered that ions are produced during crystallization process[126]. The matrix acts as a proton donor or receptor to help analyte ionization in a positive or negative mode, but also helps to prevent sample

damage from the laser pulse[127]. The most commonly used matrices suited for nitrogen (337 nm) and neodymium-doped yttrium aluminium garnet (Nd-YAG) lasers (266 or 335 nm) are 2,5-dihydroxybenzoic acid (DHB) for peptides, proteins, lipids, nuclear acids and saccharides, α-cyano-4-hydroxycinnamic acid (CHCA) for peptides, sinapinic acid (SA) for proteins and polymers, and 3-hydroxypicolinic acid (HPA) for nuclear acid and DNA. The laser spot diameter may vary from 5 to 200 μm[128].

There are three types of analyzers normally combined with MALDI: linear time of flight (TOF), reflectron TOF and Fourier transform ion cyclotron resonance (FTICR) mass analyzer. MALDI-TOF is the most simple and frequently used instrument; single charged ions are generated with a large mass-to-charge ratio (*m/z*) value. The principal of MALDI-TOF is based on accelerating ions in a constant electric field region and their separation in a field-free time-of-flight tube. While all the ions have the same energy from the electric field, they would reach the detector at different times because of their different masses. The velocity of ions depends on the mass-to-charge ratio; the smaller ions would reach the detector first and afterwards larger ions. The main advantages of MALDI-TOF are: (i) fast analysis, (ii) simple mass spectrum for analysis because most ions are singly charged, (iii) no mass range limitations in theory, while up to 100 kDa in practice, (iv) high spatial resolution for MS imaging, (v) high sensitivity, and (vi) better tolerance than ESI to the presence of contaminants, such as salts or small amounts of detergent. However, there are also some limitations: (i) inhomogeneous surface sample separation resulting in low reproducibility, (ii) interference of matrices ions in low mass region, (iii) difficulties in coupling with separation techniques, and (iv) low mass resolution of large biomolecules due to wide energy spreading during the desorption of molecules and biomolecule fragmentation.

To correct the kinetic energy distribution and to enhance the resolution, the reflectron TOF mass spectrometer was proposed by Mamyrin[129]. However, with an increase of resolution there is a commensurate decrease in sensitivity and this limits the spectra to a relatively low mass range <10 000 *m/z*.

In 1974, Comisarow and Marshall have coupled MALDI to ultrahigh-resolution (>10^5) FTICR mass analyzer to measure ion cyclotronic frequency in a magnetic field. FTMS provide a high accuracy with low as 0.01%; however, such instrument is very expensive due to the supramagnetic field needed to induce circular motion[130, 131].

The ability to generate an accurate information regarding protein sequence, identification and PTMs make MALDI-MS a very popular tool for proteomics. MALDI-MS can also tolerate a moderate amount of salt and detergents, allowing characterization of proteins without their purification. Protein identification can be facilitated using tandem mass spectrometry (MS/MS), where the analysis of the protein's proteolytic peptide fragments takes place. Parent peaks of interest can be selected first by a low-resolution TOF; then the selected ions fragmented *via* collision-induced dissociation (CID) are analyzed by a second high resolution TOF to provide information about the primary structure and modifications of sample.

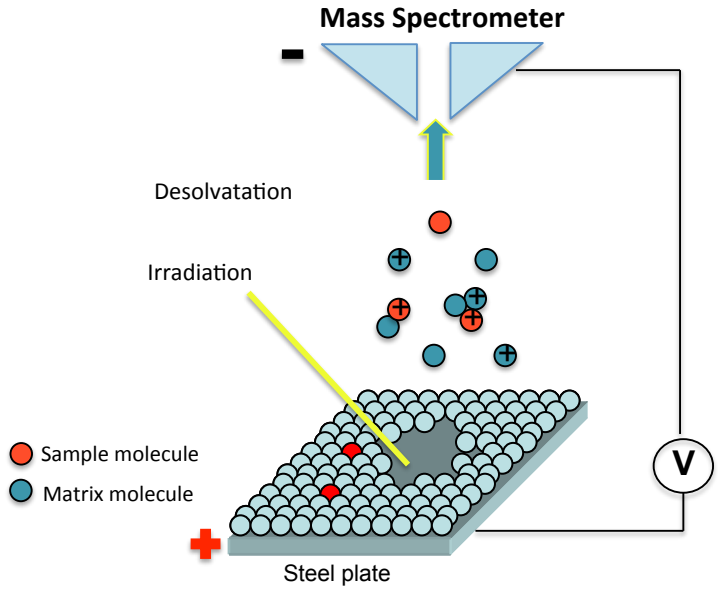

Figure 1.8. The principle of matrix-assisted laser desorption/ ionization time of flight mass spectrometer.

Protein identification and peptide mass fingerprinting (PTM) are the main applications of MALDI-MS, due to its high sensitivity. As well, MALDI-MS is used for analyses of inorganic compounds; most of the studies were focused on characterization of organometallic and inorganic coordination complexes and their use as potential matrixes. Tanaka *et al.* used cobalt powder as the matrix[132]. 11 different metal powders were tested by Niwa *et al.,* showing that ions up to 2000 Da can be observed[133]. MALDI-MS is not useful for the analyses of organic compounds; however, it was applied to the analyses of fullerenes due to their thermal stability[134]. This soft ionization technique has been found to be a powerful tool for the characterization of polymers[135]. MALDI-MS was used for the detection of disease biomarkers of cancer[136], Alzheimer[137, 138], rheumatoid arthritis[139] and allergens[140],

in mass spectrometry imaging[141], as well as for many other applications reported in literature[142-144].

3.2. Electrospray ionization mass spectrometer

In 1750, Nollet described the phenomenon, when the spray from electrified metallic vessel took place. In 1882, Reyleigh estimated the maximum amount of charge, which a liquid droplet can sustain, later called as "Reyleigh limit"[145]. Zeleny was the first to describe the behavior of droplets at the end of the capillary[146]. Only in 1989, an ionization technique to generate multiple charged species named electrospray ionization (ESI) was introduced by Fenn and co-workers[116, 147]. Since then, ESI-MS became one of the most widely used ionization techniques, through which it is easier to obtain evidence towards protein three-dimensional structures, including amino acid sequence and PTMs.

In electrospray ionization, the sample in solution is injected through a silica capillary at a flow rate in the range of 1-500 μl/min (nanoESI typical liquid flow rates: 10 nL/min - 1 μL/min) into the ESI-MS while an electric field in the range of 2-5 kV is applied to the capillary. Depending on the sample, either a negative or a positive voltage can be applied, which generates gas-phase cations or anions, respectively. Since the strong electric field is applied, the liquid is extruded from the capillary, which is called "Taylor cone" (see Figure 1.9)[148], and the dispersion of the sample solution into an aerosol of highly charged droplets occurs. The charged droplets, decreasing in size through solvent evaporation, are supported by a co-axial flow of neutral gas, known as sheath gas flow (dry N_2), which can also aid nebulization. The charged ions are finally formed. Two models are usually considered to account ion formation: the charge residue model (CRM), proposed by Dole, suggesting that due to solvent evaporation large droplets are divided to much smaller ones until it would consist of only single ions[149]; while, the ion evaporation model (IEM) by Iribarne and Thomson, proposes that the charge density of the droplet could become large enough to result in the release of ions directly from droplet surfaces[150, 151]. IEM controls mainly the formation of small ions (smaller than 10 nm), whereas CRM facilitate the foundation of large ions.

In electrospray, the molecules are observed as quasi-molecular ions created by protonation or deprotonation. For large biomolecules, multiply charged ions are

observed with many charge states. The number of charged molecules depends on the mass, properties of the molecule, as well as the solvent utilized. However, many peaks for single molecules could be detected [152].

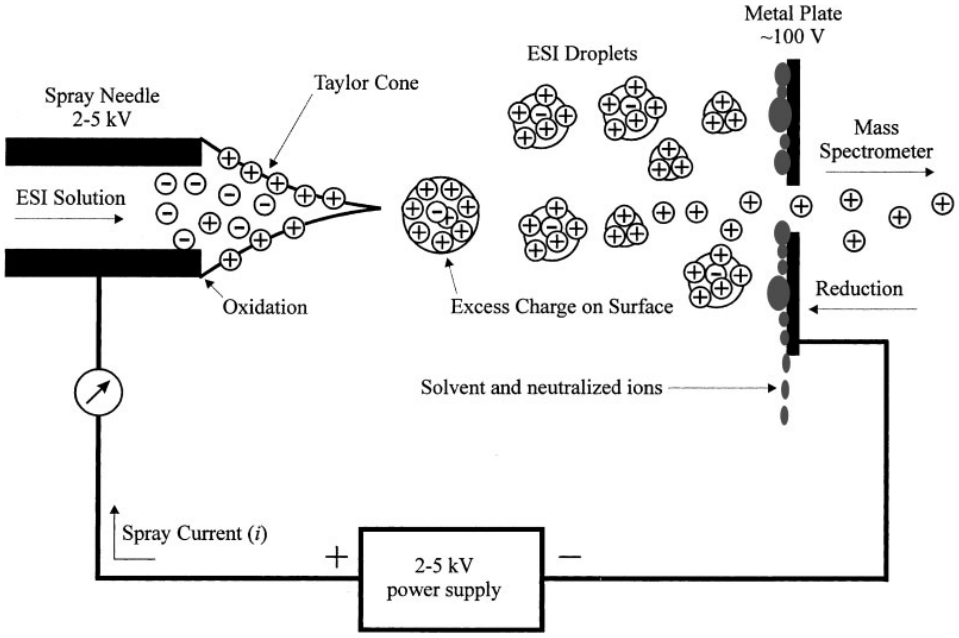

Figure 1.9. Schematic representation of electrospray ionization (ESI) process. High voltage is applied to the needle with analyte solution pumped through. The excess of positive charge is formed on the Taylor cone surface as a result of electric field gradient developed between ESI needle and counter electrode (i.e. mass spectrometer). Charged droplets occur at the tip of the Taylor cone, evaporate and move towards the entrance of mass spectrometer to produce charged molecules that can be analyzed for their mass-to-charge ratio. Reprinted from Cech *et al*., Mass Spectrometry Review, 20, 362, 2001[153].

ESI-MS provides fast and comprehensive information concerning the quantity and structural properties of analyzed molecules. One main difference of ESI-MS compared to MALDI-MS is that in MALDI mainly the singly charged ions are generated, while in ESI multiply charged molecules are produced. With this property, the ESI source producing low *m*/*z* of each biomolecule regardless of its molecular mass can be coupled to many mass analyzers; not only TOF and FTICR, but also ion trap, quadropole and orbitrap. However, due to the presence of multiply charged ions, the mass spectra obtained are more complex. Also, ESI provides a very high sensitivity and can be easily coupled to liquid chromatography or capillary electrophoresis[154]. CE-MS coupling was firstly developed by Smith *et al.* and became a powerful tool providing rapid, efficient, and sensitive analyses of complex biological mixtures[155]. LC-ESI-MS has become the most efficient and widely used

technique of protein sequencing in bottom-up proteomics[156]. The proteins/peptides are separated by strong cation exchange (SCX) LC and RPLC, which also help to remove the salts before MS analysis[157].

ESI-MS allows identification of different bimolecular complexes, such as protein-protein, protein-metal ion, and protein-drug complexes[158, 159]. Since femtomoles of the sample are enough for the analysis, ESI-MS become an important tool in clinical laboratory for structural analysis of metabolic complexes[160].

3.3. Ambient ionization mass spectrometry

Ambient ionization mass spectrometry is a concept of MS with sample ionization under ambient conditions (standard temperature and pressure) with minimal or without any sample pretreatment[161]. A wide variety of mass spectrometers can be coupled to the ambient ion source.

Several new ambient ionization techniques have been developed based on ESI: (i) electrospray laser desorption ionization (ELDI) MS for analysis of nonvolatile compounds from the surface of a solid sample[162], (ii) laser ablation electrospray ionization (LA-ESI) where the compounds are analyzed by affecting the sample surface with heated stream of N_2 gas[163], (iii) direct electrospray probe (DEP) for rapid analysis of dirty sample in extremely low volumes[164], (iv) extractive electrospray ionization (EESI) with the one sprayer nebulizing solution into the other to produce charged droplets[165] and (v) desorption electrospray ionization (DESI) [166].

DESI is probably one of the most popular ambient techniques, developed in 2004 in the group of Prof. Cooks[166] representing the combination of electrospray and desorption methods (see Figure 1.10). Electrically charged droplets are used to create macroscopic liquid layers on the sample surface. Generated ions from the sample travel afterwards to MS inlet through air.

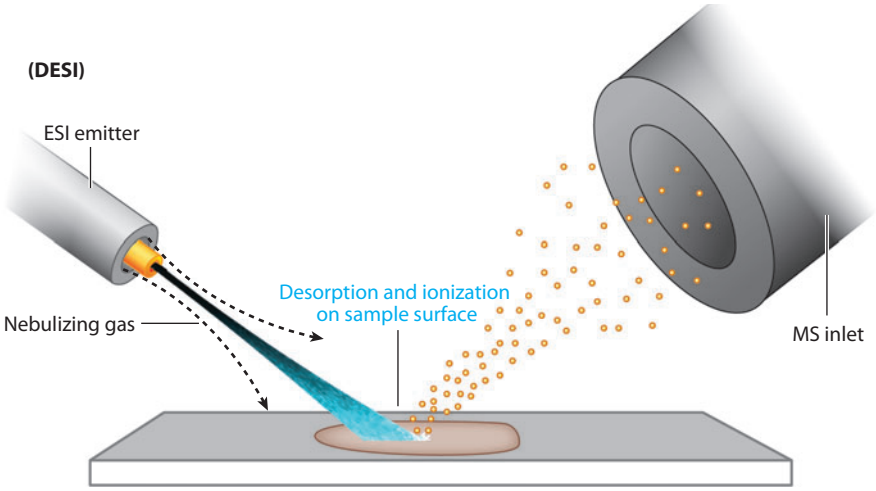

Figure 1.10. Schematic representation of desorption electrospray ionization technique. Reprinted from Huang *et al.,* Annual Review of Analytical Chemistry, 2010[167].

Due to the difference in the ionization mechanism, DESI can be applied to the analysis of low and high molecular weight molecules[168], as well as for a wide range of applications in different fields, such as protein/peptide analysis, drug detection[169], explosives detection[170], and plant tissue analysis[171]. As demonstrated on various substrates, like brain and liver tissues[172], the main application of DESI is imaging. Previously, the method was limited to a spatial resolution of typically 180-220 μm [173]; however, this has since been improved up to an average of 40μm[174] with the best resolution reported as 10 μm using nanospray DESI[175].

In 2005, Laramee and Cody developed another ambient technique that is probably the most wide spread, named Direct Analysis in Real Time (DART), in which no sample preparation is required and the ionization takes place directly on the sample surface[171]. DART uses a solvent-free heated gas stream to carry the ionizing atoms that have been formed upon interaction of the electronically exited atoms with the atmosphere[172]. DART-MS can be applied for rapid analysis of a wide variety of single compounds or complex mixtures, with more then 260 applications having been presented so far[173].

Ambient ionization techniques can be considered as a new branch of mass spectrometry. These approaches drastically improve time of analysis and broaden the scope of the sample types, which can be analyzed by MS. One of the main advantages of ambient techniques is that minimal or no sample preparation is needed, meaning that the sample of interest is analyzed in its native state. That makes ambient ionization MS a progressive field with perspective future.

4. Thesis outline

In the context of pre-fractionation tools for proteomics, this work deals mainly on the development of new analytical methods for efficient and fast separation of proteins and peptides from complex biological samples. Another goal of the present work is to find a way of identifying the samples from the surface by contactless method using mass spectrometry.

In **chapter II,** a multi-electrode setup for protein OFFGEL electrophoresis that significantly improves protein separation efficiency has been developed. Here, the electric field is applied by segments between seven electrodes connected in series to six independent power supplies. The aim of this strategy is to distribute evenly the electric field along the multi-well system, and, as a consequence, enhance electrophoresis in terms of separation time, resolution and protein collection efficiency, while minimizing the overall potential difference and therefore the Joule heating. The performances were compared to a standard two-electrode setup for OFFGEL fractionation of a protein mixture, using UV-Vis spectroscopy for quantification and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) for identification. The electrophoretic separation process was simulated, and optimized by solving the time-dependent Nernst-Planck differential equation.

Chapter III is dedicated to purification and desalting of protein and peptide samples by three-well OFFGEL electrophoresis with immobilized ultra-narrow pH gradient gels, as a fast preparative strategy for proteomics. The gist of this strategy is to separate the proteins and peptides according to their isoelectric point and to isolate those of a given p*I* value equal to the mean pH value of the gel. The present approach has been demonstrated both on protein mixtures and a digested *Escherichia coli* protein extract. UV-Vis spectroscopy, MALDI-MS, SDS-PAGE and LC-MS/MS were employed for the quantitative and qualitative characterization of the separation results. The electrophoretic methodology has been simulated using COMSOL Multiphysics software, which employs the finite element method so that geometric effects are represented.

Chapter IV describes the concept of a compact device, which allows direct electro-elution of fractionated charged molecules from an ultra-narrow pH gradient polyacrylamide gel. The goal of the present system is to provide protein purification,

complex mixture fractionation and direct coupling with matrix-assisted laser desorption ionization (MALDI) or electrospray ionization mass spectrometric analysis. A high potential difference is applied that forces the proteins to migrate into a liquid droplet directing analyte species further towards the detection unit by liquid flow. An average sample recovery of 90% was achieved using a protein test mixture. The preliminary results for such an electro-elution device show that fast and effective protein elution can be achieved but improvements in the design is still required.

In **chapter V** gel electrophoresis has been used for decades as a high-resolution separation technique for proteins and protein isomers, but has limited in the coupling with MS because of low throughput and poor automaticity compared with LC-MS. In this work, we have developed an ambient ionisation strategy, Electrostatic Spray Ionisation (ESTASI), for *in-situ* ionisation of proteins or peptides inside a surfactant-free polyacrylamide gel. The samples can be first separated by isoelectric focusing in a gel and then quickly *in-situ* detected by scanning the gel with the electrostatic spray ionisation mass spectrometry. With this strategy, nanograms of proteins or peptides inside a band are enough to be ionised for MS detection. This method for protein/peptide spots visualization is sensitive providing sample molecular weight information whilst avoiding spot staining and chemical extraction procedures that can introduce contaminants and sample loss. Proof-of-principle results have demonstrated that the electrostatic spray ionisation can produce sample ions from a complex background and with a spatial resolution matching the isoelectric focusing; therefore, a good choice to couple directly isoelectric focusing gel electrophoresis with mass spectrometry.

Chapter VI introduces ESTASI used to analyze directly samples sprayed or deposited on different types of paper. The paper is placed on a plastic support behind which is an electrode connected to a pulsed high voltage source. A modified transfer capillary is used to “sniff” the sample for mass analysis. The paper ESTASI-sniffing is used for analysis of a model perfume and several commercial perfumes. This strategy yields a rapid fingerprinting characterization of fragrances, avoiding time-consuming sample-preparation steps, and thereby performing a rapid screening in only a few seconds.

Chapter VII presents standard addition strips for the quantitative determination of caffeine in different beverages by Electrostatic Spray Ionisation mass spectrometry (ESTASI-MS). The gist of this approach is to dry spots of solutions of caffeine of

different concentrations on a polymer strip, then to deposit a drop of sample mixed with an internal standard, here theobromine on each spot and to measure the mass spectrometry signals of caffeine and theobromine by ESTASI-MS. This strip approach is very convenient and provides quantitative analyses as accurate as the classical standard addition method for quantitative analysis.

In **chapter VIII** the direct analysis and desalting of samples from ZipTip by ESTASI-MS is presented. The limit of detection for proteins and peptides was estimated by spraying the samples from micropipette tip. The samples containing different amounts of salts were also evaluated by ZipTip-ESTASI and compared with the results obtained by using the commercial source of ESI and after spraying the solutions from the insulating plate by standard ESTASI-MS. It was shown that ZipTip-ESTASI-MS provides a direct, fast and convenient method, which can be applied for proteome analysis, since the presence of salts has been a persistent problem for ESI-MS.

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Chapter II.

Segmented Field OFFGEL® Electrophoresis

Based on E. Tobolkina et al. Electrophoresis, 2012, 33 (22), 3331-3338.

1. Introduction

Proteomics, the study of proteins involved in metabolic pathways and their interaction presents three main challenges to any analytical methodology[1, 2]: i) high sensitivity, since protein concentrations vary over a wide dynamic range and since low abundant proteins play a relevant role in most biological processes[3, 4]; ii) high resolution power to separate, extract and/or distinguish one or a group of proteins from a complex matrix; and iii) a reasonably short experimental time. Improving any of these three points can certainly speed-up research and discovery in proteomics. Two-dimensional gel electrophoresis (2D-PAGE) has been the workhorse strategy for protein analysis during the last 30 years[5]. This approach is based on the orthogonal separation of proteins according to their isoelectric point (p*I*) in the first dimension and their molecular weight in the second dimension[6-8], allowing the separation up to thousands of proteins. However, 2D-PAGE suffers from a number of inherent limitations such as being time consuming, providing a low protein detection sensitivity after separation, or stemming from the co-migration of proteins that introduces artifacts and makes the visualization of protein spots sometimes rather difficult. Moreover, proteins with extreme p*I* values (*i.e.* below 3 and above 10) or extreme molecular weight might be dismissed during the separation. Therefore, new strategies that alleviate the drawbacks presented by 2D-PAGE, or that become an alternative or complementary strategy for high throughput protein analysis, are needed.

For instance, in 2002 Ros *et al.* introduced a new concept for protein separation so-called OFFGEL electrophoresis based on a preparative approach of IEF electrophoresis[9, 10]. The idea behind is simply to place a frame containing several wells of identical dimensions over a hydrated immobilized pH gradient (IPG) gel, in such a way that the pH gradient is divided between the wells, but without loosing connection through the whole gel. Then, an equivalent volume of buffered sample solution is loaded in each well (*i.e.* on top of the gel), and an electric field between two electrodes localized at the extreme sides of the frame is applied. As a consequence, the proteins present in the added solution are forced to migrate through

the gel from one well to another according to their p*I*s. Once the proteins have been iso-electrofocused in one well (*i.e.* in the well where the pH is close to the protein p*I* value), the proteins are distributed between the gel below and the solution above, allowing the collection of protein fractions. Thanks to the different advantages that OFFGEL provides to proteomics (*e.g.* allowing an easier coupling of protein electrophoresis with sensitive protein detection techniques such as mass spectrometry)[11] it is now a widely used technique. A variety of samples have been analyzed by using OFFGEL electrophoresis, including *Escherichia coli* [12], human plasma [13] and eukaryotic samples[14]. Despite of this, protein electrophoresis can be further improved by changing the paradigm of electric field application[15-17]. Usually, electrophoretic separations employ only two electrodes (*i.e.* located oppositely at each side of the gel) to apply an electric field to the separation media, which depending on its magnitude will affect drastically the protein separation efficiency (*e.g.* resolution power and experimental time), and also the protein collection efficiency in OFFGEL electrophoresis, for instance. Therefore, one strategy for improving protein electrophoretic separations and specially OFFGEL electrophoresis is to apply a higher and more homogeneous electric field across the whole system.

In the present study, a multi-electrode setup is introduced for OFFGEL electrophoresis, to provide a more efficient application of the electric field. The multi-electrode setup consists of a lid with seven platinum electrodes placed over an OFFGEL frame with seven wells with, for demonstration, one electrode in each well. The separation of a mixture of five proteins by OFFGEL electrophoresis in both a multi-electrode and in a two-electrode formats shows that the multi-electrode setup yields a better protein separation resolution, as well as, a higher protein collection efficiency in a shorter time. Furthermore, analysis of an *E. coli* extract has demonstrated that the proposed methodology can successfully be applied to the fractionation of complex samples. Additionally, numerical simulations were performed to describe both electric field distribution and protein migration inside multi-electrode and two-electrode OFFGEL separations corroborating the experimental results.

2. Materials and methods

2.1 Materials

IPG gels (Immobiline® Drystrips, linear pH range from 3.0 to 10.0, 7 cm length) and silver and Coomassie blue staining kit protein visualization were purchased from Amersham Biosciences (Uppsala, Sweden). β-Lactoglobulin AB (p*I* 5.1) from bovine milk, α-lactalbumin from bovine milk type I (p*I* 5.02), cytochrome C from horse heart (p*I* 9.6), myoglobin from horse skeletal muscle (p*I* 7.0, 7.4) and ribonuclease A (RNase A) from bovine pancreas (p*I* 9.45) were obtained from Sigma-Aldrich (Schnelldorf, Switzerland), as well as, sinapinic acid, acetonitrile, trifluoroacetic acid, methanol and acetic acid of the purest grade (>99.9 %). Deionized (DI) water was purified by an alpha Q Millipore system (Zug, Switzerland) and used in all aqueous solutions. The UV-visible absorption spectra were obtained with a standard spectrophotometer (Perkin Elmer, model Lambda XLS+) using quartz cells with a path length of 1 cm. A calibration BCA protein assay kit for determining protein concentrations was obtained from Thermo Scientific (Rockford, USA). Common OFFGEL electrophoresis was performed with the Agilent 3100 OFFGEL fractionator (Waldbronn, Germany). For the multi-electrode setup, six power supplies from Fug (Switzerland) were used.

2.2 OFFGEL electrophoresis using two- and multi-electrode setups

Immobiline® Drystrips with a 7 cm length and a pH gradient from 3 to 10 were used as gel media for OFFGEL electrophoresis, as it most suitably fit the length of the multi-electrode device. After re-swelling in water the IPG strip for 1 hour, it was placed on a flat surface and then covered with the multi-well frame. All wells of the device were filled with DI water (*ca.* 150 μL), except the middle well (No. 4 in Figure 1) where 150 μL of a solution containing β-lactoglobulin, α-lactalbumin, cytochrome C, myoglobin and RNase A (the concentration of each protein was 26 μg/mL) was added. For OFFGEL electrophoresis with two electrodes, a commercial Agilent 3100 Fractionator was employed and the anode and cathode platinum electrodes were placed outside each opposite border of the plastic frame. Two electrodes were placed at the extremes of the multi-well frame in a two-electrode setup, using one of the

power supplies from the multi-electrode system. In the case of the multi-electrode setup, one platinum electrode was placed inside each well in the OFFGEL frame allowing the use of 7 electrodes powered individually, but connected in series with 6 power suppliers (see Figure 2.1). The electrode placed in the first well of the multi-electrode setup was connected to the ground (0 V) output of each power supply, acting as the more negative electrode in the whole system. The second electrode was connected to the high tension output of the first power supply and placed inside the second well, while the third and subsequent electrodes are connected to the high tension terminals of their respective power supply and placed in the succeeding wells (see Figure 2.1). Since all the Pt electrodes are connected in series, the potential difference along the system increases additively, providing a more positive electrode each time at the right side. In the present study, a progressive increment of the potential difference applied between neighbouring electrodes was employed. Both the voltage and the current were monitored during all the experiments.

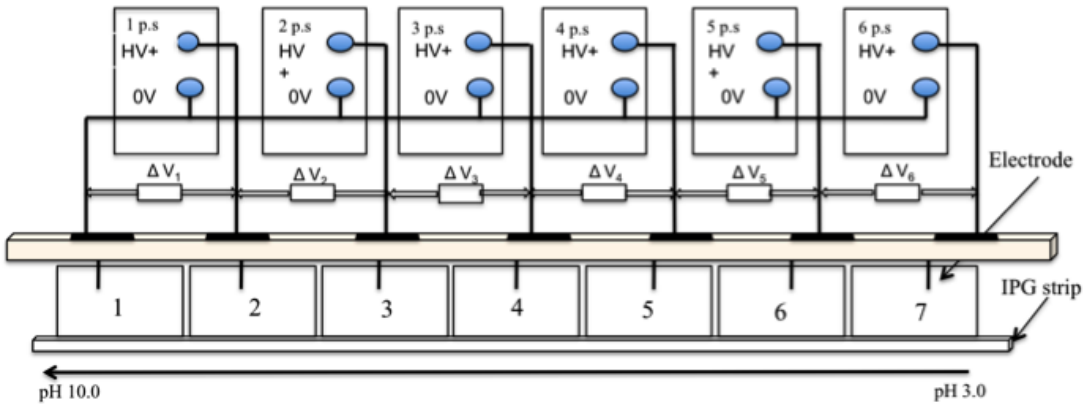

Figure 2.1. Schematic illustration of the multi-electrode setup, consisting of seven electrodes connected in series with several power suppliers. The sample is placed in a chamber and covered with a lid with seven Pt electrodes.

2.3 Soluble *E. coli* protein extract preparation

An over-night 200 mL culture of *E. coli* (strain DH5a) was collected by 10 min centrifugation at 5000 *g* and 4°C. The cell pellet (0.6 g wet weight) was re-suspended in 3 mL of lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA) and cells were disrupted by sonication (10 x 10 pulses of 1 s at 30 W). Cell debris were removed by 10 min centrifugation at 2000 *g*. Ultracentrifugation (1 h at 100'000 *g* at 4°C) was

applied to the total cell extract to remove membranes and membrane-bound proteins. The supernatant was collected and was considered as the soluble fraction of *E. coli* proteins.

2.4 MS analyses

The MS analyses were performed on a Microflex MALDI-TOF instrument (Bruker Daltonics) equipped with a 337 nm nitrogen laser. 1 μL of the extracted protein solution was deposited on a steel target plate with 1 μL of sinapinic acid (SA) matrix (15 mg/mL Sinapinic acid in 50% acetonitrile, 0.1% Trifluoroacetic acid and 49.9% water) and left to dry at room temperature.

2.5 Finite element simulations

Numerical simulations were performed using finite element package COMSOL Multiphysics (version 3.5a) installed on a Mac Pro with four 2.66 GHz central processing units and 9.8 GB of RAM operating under Linux Ubuntu 8.04 platform. The mass-transport of charged species (*i.e.* proteins) was simulated in a two-dimensional computational domain of an OFFGEL setup (see Figure 2.1) utilizing Nernst-Planck without electroneutrality and conductive media DC application modes from Chemical Engineering and AC/DC modules, respectively. Numerical resolution of corresponding partial differential equations (PDEs) was obtained using direct solver UMFPACK in transient mode with relative error tolerance 10^{-6} and taking time steps from solver. In order to reduce computational efforts the solution was computed sequentially, *i.e.* solving mass-transport equations on top of the stored solution containing electric field distribution. The mesh size was adjusted down to the value of 10 μm at the corners of the wells and electrode edges.

3. Results and discussion

3.1 Numerical simulation of protein migration and electric field distribution in the OFFGEL device

The validity of a multi-electrode approach for OFFGEL fractionation was examined simulating electrical properties of the gel and modeling protein separation for the OFFGEL setup geometry shown in Figure 1. We have used finite element simulations for numerical analysis of Nernst-Planck equation (eq. 2.1) describing mass-transport of species i [18] as

$$\frac{\partial c_i}{\partial t} + \mathrm{div}\left[-D_i \nabla c_i - \frac{z_i F}{RT} D_i c_i \nabla \phi\right] = 0 \tag{2.1}$$

and Laplace equation (eq. 2.2) determining electric field distribution within the computational domain

$$\nabla(-\sigma \nabla \phi) = 0 \tag{2.2}$$

Herein c_i, t and ϕ are the dependent variables, specifying concentration, time and electric potential, while D_i, z_i, F, R, T and σ are constant values denoting diffusion coefficient, charge number, Faraday constant, gas constant, temperature and electrical conductivity of the medium, respectively (see details in Appendix 2.0).

The Immobiline gel in our model is considered as a conductive media with an electrical conductivity determined by free, non-covalently bond ions in the matrix[19], while the sample is assumed to have no influence on electrical properties of the domain. Therefore, the conductivity is given as the function of local pH value, determined by the contribution from H^+ and OH^- ions

$$\sigma = \frac{F^2}{RT}\left(D_{H^+} 10^{(3-pH)} + D_{OH^-} 10^{(-14+pH)}\right) \tag{2.3}$$

Although the conductivity variations within the gel arising during protein focusing have been reported[20], the quantitative description of these experimental observations are still unavailable and therefore this simple approximation of σ has been used in the present work. This function (see Figure 2.2a) has a pronounced minimum value close to neutral pH resulting in a very resistive region at pH 5 – 9

giving rise to a sharp local increase of the electric field. As a consequence, the overall driving force for a charged species remains at very low values along the gel except in the low conductivity zone formed in the middle as shown on Figure 2.2b. However, the significant improvement of an electric field distribution is observed when the potential difference is applied in a segmented manner using multi-electrode arrangement (see Figure 2.2b). As compared to a standard two-electrode configuration, the driving electrophoretic force is spread along the gel in a uniform manner that comprises lows and peaks of comparable intensity. Most likely, such changes in electric field distributions arise from variations of current density under well compartments and in between them.

Another advantage of a multi-electrode system is the possibility to tune the potential program (*i.e.* the potential differences applied to each electrode) for electrophoretic separation of a particular sample mixture keeping overall potential difference constant. Figure 2.2c illustrates this feature exhibiting the simulated electric field distribution for the potential program adjusted for a separation of proteins with isoelectric points in acidic medium (see details in Appendix 2.0). As can be seen, the electric field lows and peaks reach higher values than in general case (Figure 2.2b) in the acidic region of the immobilized pH gradient gel resulting in faster and more efficient separation of target proteins (*vide infra*).

In order to compare the IEF efficiency for the common OFFGEL two-electrode arrangement with the multi-electrode configuration, the isoelectric focusing of a test protein (α-lactalbumin) was simulated. Following previous numerical simulations for OFFGEL separations presented by Lam *et al.*[21], the net charge of a protein could be expressed, for instance, via Henderson-Hasselbach relation (eq. 2.4).

$$z(\mathrm{pH}) = -\sum_{i \in A^-} \frac{1}{1 + \frac{10^{-\mathrm{pH}}}{K_i}} + \sum_{i \in A^+} \frac{1}{1 + \frac{K_i}{10^{-\mathrm{pH}}}} \tag{2.4}$$

where K_i is the ionization constant of the ionizable group of the corresponding amino acid, while A^- and A^+ denote the negative and positive charge of amino acids. The positive charge can be provided by histidine (H), arginine (R), lysine (K) and N-terminus. The negative charges are given by tyrosine (Y), aspartate (D), cysteine (C),

glutamate (E) and by the charge of C-terminus[22]. It is important to notice that by employing this simple approach for the protein charge calculation some limitations can be introduced, since slightly variations from the use of macroscopic and microscopic dissociation constants can take place[23]. However, the aim of the present work is not to calculate precisely the p*I* of the proteins but to demonstrate the advantages that the multi-electrode setup offers to OFFGEL electrophoresis. The simulated titration curve (net charge *vs* pH) of α-lactalbumin depicted on Figure 2.2d has a well-defined isoelectric point (p*I*= 5.02) and has a sufficient slope at p*I* value that was shown to be a key parameter in focusing dynamics; hence, a fast and efficient separation of this protein is expected.

Figure 2.2e displays the simulated concentration field of α-lactalbumin after IEF with both two- (bottom) and multi-electrode (top) configurations. The differences in separation efficiency are clear: in a multiplex arrangement the test protein is fairly focused mostly in one well with a collection efficiency of 97%, in contrast to the 45% achieve in two-electrode (and also standard OFFGEL) setup where α-lactalbumin was dispersed between three wells. Finally, the time required for completely focusing α-lactalbumin by using multi-electrode setup was examined for three different voltage programs with an overall potential difference of 1 V (*i.e.* optimized potential program for general protein mixtures, adjusted voltage program for separation of proteins with acidic p*I* and common two-electrode OFFGEL) (see details in Appendix 2.0). The fastest IEF process was achieved within 418 s for the adjusted voltage program for separation of proteins with acidic p*I*, while focusing with the optimized voltage program for general protein separations and with the common two-electrode setup took 1.26 and 62.17 times longer, respectively. As suggested by these numerical results, the multiplexed electrodes approach for IEF electrophoresis allows for faster, more efficient and quantitative electrophoretic separations due to the enhanced electric field distribution. Additionally, when working with known protein mixtures that present mainly acidic p*I*s, for instance, or that due to their molecular weight will limit the required time for focusing all the proteins, a special voltage program can be designed.

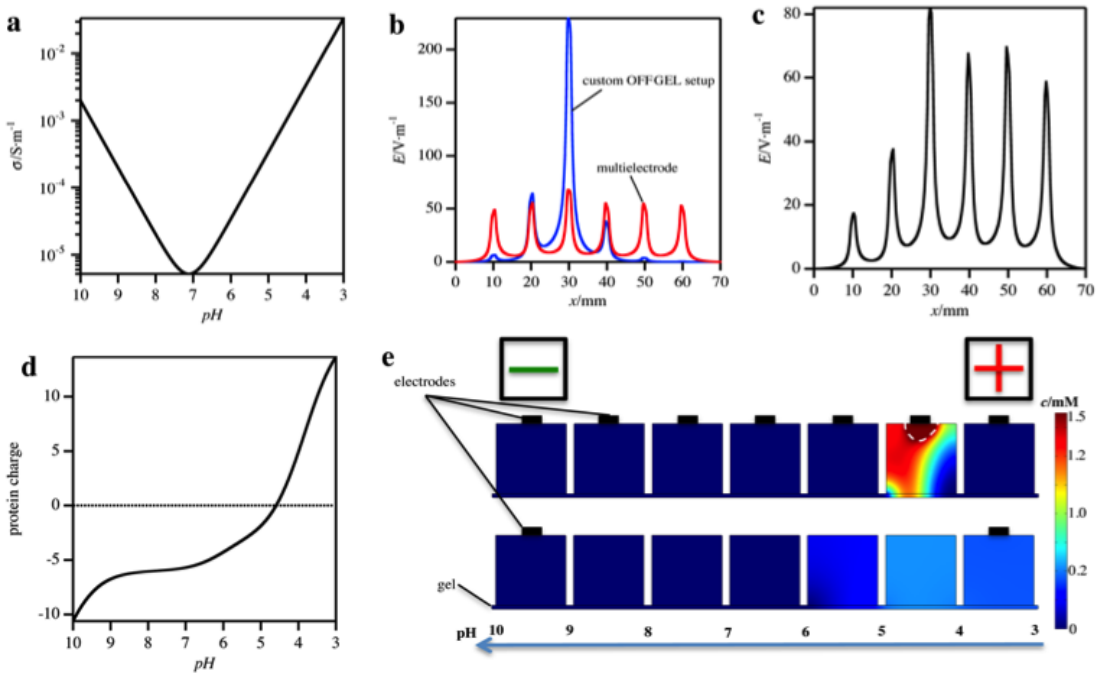

Figure 2.2. Finite element simulations of IEF using the multi-electrode approach. a) Electrical conductivity (given in a logarithmic scale) in the immobilized pH gradient gel according to eq 2.3. Simulated electric field distributions along a gel medial cross-section for b) a multi-electrode setup with an optimized voltage program for general protein mixture separations, a two-electrode setup for common OFFGEL and c) a multi-electrode with an adjusted voltage program for the separation of proteins with acidic p*I*. d) calculated titration curve (overall charge *vs* pH) of α-lactalbumin. e) calculated protein concentration field for multi- (top) and two-electrode (bottom) arrangement (see scale bar on the right). The electrodes are schematically depicted on top of the well's compartments, "+" and "−" signs reveal the direction of the applied electric field; pH gradient scale is shown on the bottom.

3.2 OFFGEL electrophoresis with a multi-electrode setup

A mixture of five different proteins (*i.e.* β-lactoglobulin, α-lactalbumin, cytochrome C, myoglobin and RNase A, 26 μg/mL each) was separated by OFFGEL electrophoresis with the multi-electrode setup shown in Figure 2.1. For this protein separation an IPG gel with a pH range from 3 to 10 was employed, and covered with a multi-well system. The solution containing the protein mixture was added to the middle well (*i.e.* No. 4 in Figure 2.1), since according to the numerical simulations a higher electric field is found in this region forcing a faster migration of the proteins at the first stages of the electrophoretic process. However, in principle the sample can be added to any well or to all of them and the same result should be obtained if the proper experimental time is used to achieve a complete separation. Then, the multi-electrode setup was placed over the multi-well frame. The applied potential difference

between the different neighbouring electrodes (ΔV_i) can be seen in Figure 2.1, where a clear progressive increment in ΔV_i was adjusted in such way that ΔV_i is always lower than ΔV_{i+1} by a value of 25 V. Since all the seven electrodes employed in the multi-electrode setup were connected in series (see experimental part), the total potential difference applied through the whole system is equal to the sum of each ΔV_i and therefore equal to 675 V. The employed voltage program corresponds not to the optimized voltage program for a general protein mixture, but to the adjusted one for sample employed in the present work (*vide supra*). OFFGEL electrophoresis experiments with the multi-electrode setup were performed under these conditions during a period of 3 hours (see Table 2.1), time after which the solution over the gel was collected and analysed by MALDI-MS and UV-vis spectroscopy.

Figure 2.3 shows the mass spectra of fractions collected from wells No. 1, 3, 5 and 6 where all the added proteins where found after electrophoresis, since no protein presence was observed in the mass spectra of fractions from wells No. 2, 4 and 7 (see Figure 2.4). According to the results shown in Figure 2.3, all the proteins migrate completely from the middle well according to their p*I* and reached the expected position (*i.e.* well or wells) where the pH of the gel is close to their respective p*I* as seen in Table 2.2. For instance, α-lactalbumin was extracted from wells No. 5 and 6, as its p*I* is situated on the border between these two wells. The results shown in Figure 2.3, demonstrates that the multi-electrode setup offers to OFFGEL electrophoresis the possibility to achieve a complete protein separation in a short period of time (3 hours), simply by applying a more homogeneous electric field as described with the numerical simulations. Moreover, the potential difference applied is lower in comparison to the one usually employed in "two-electrode" OFFGEL electrophoresis (*vide infra*).

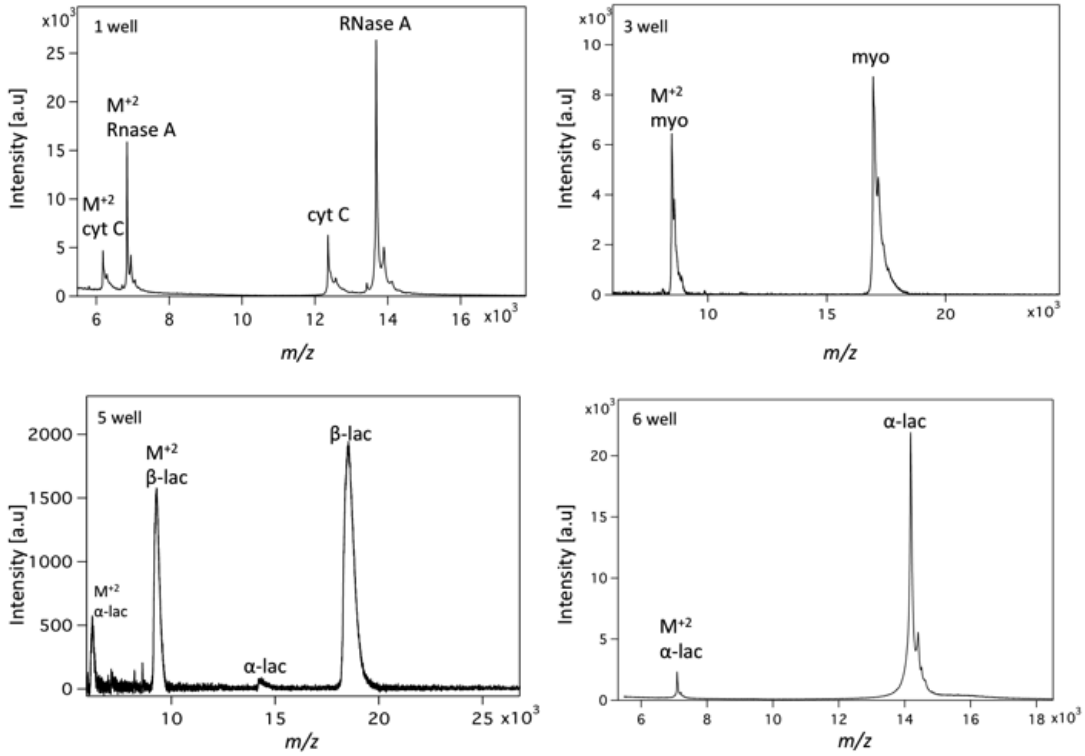

Figure 2.3. Mass spectra of fractions from wells No. 1 (a), 3 (b), 5 (c) and 6 (d) taken after OFFGEL electrophoresis performed with the multi-electrode setup. The samples were deposited on a steel target plate with sinapinic acid (SA) matrix.

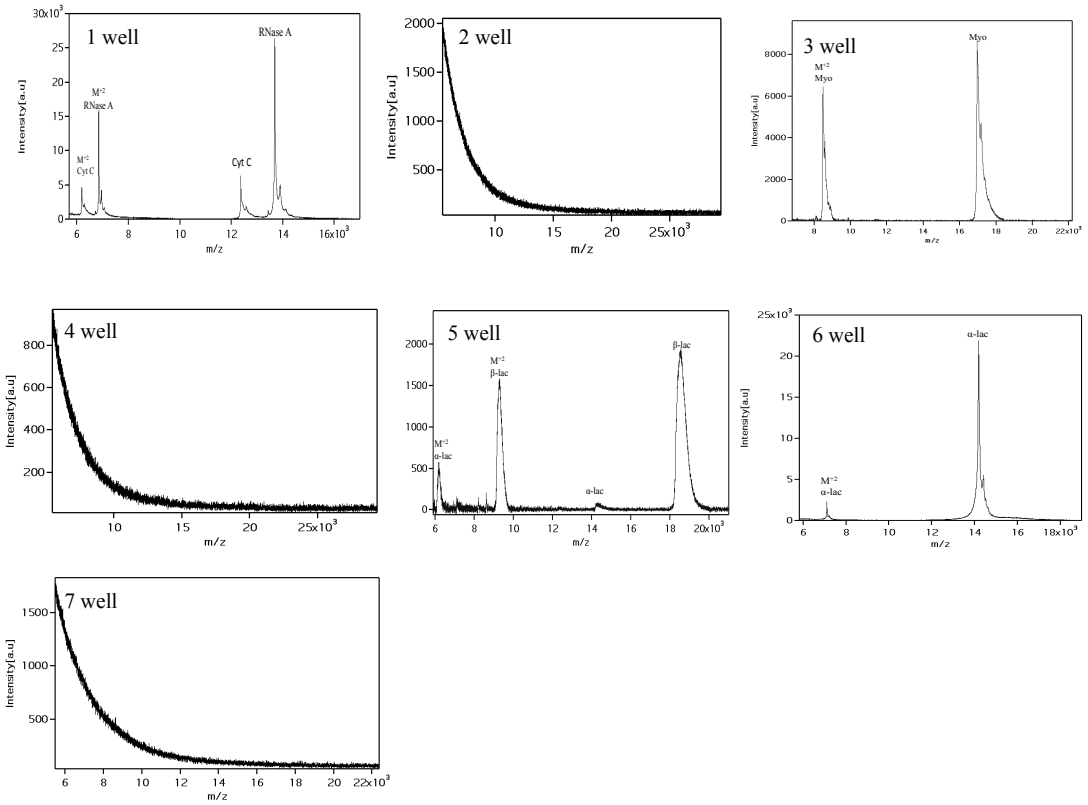

Figure 2.4. Mass spectra of fractions taken after OFFGEL electrophoresis performed using multi-electrode setup, from each of the seven wells and deposited on a steel target plate with sinapinic acid (SA) matrix.

In addition to the MALDI-MS analysis, UV-vis spectroscopy was employed to quantify the amount of protein collected after electrophoresis and therefore establishes the effect of the multi-electrode setup on the protein collection efficiency of OFFGEL. The results are summarized in Table 2.3. Basically 100 % recovery was obtained for RNase A and cytochrome C (well No. 1), Myoglobin (well No. 3) and β-lactoglobulin (well No. 5). α-lactalbumin was 38% extracted from well No. 5 and 62% extracted from well No. 6. These results indicate that 100% of all the proteins after OFFGEL electrophoresis were found in the solution and no protein was present in the gel when a multi-electrode setup was used. The latter was confirmed by the no visualization of proteins after Coomassie blue staining of the gels employed for OFFGEL electrophoresis when using the multi-electrode setup (see Figure 2.5).

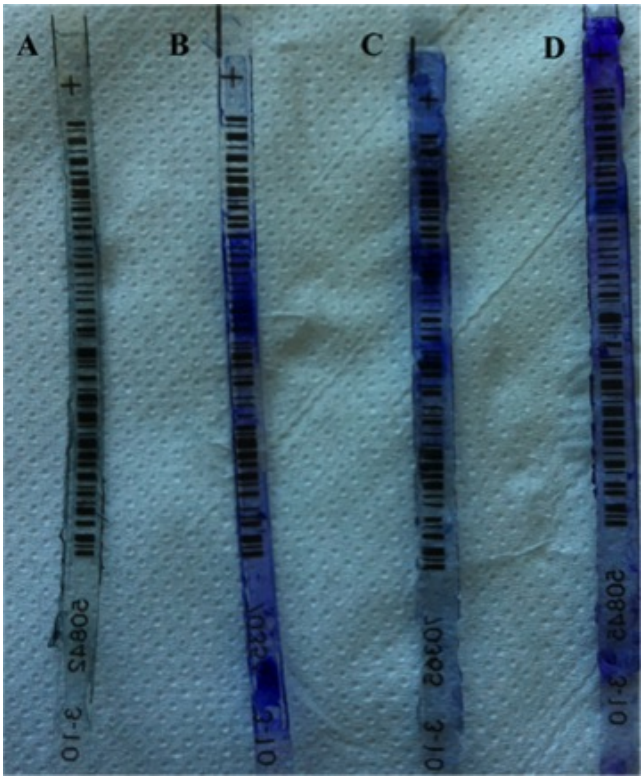

Figure 2.5. Coomassie blue stained gel strips after OFFGEL electrophoresis performed with A) Multi-electrode setup B) Two-electrode setup (using multi-electrode device) C) Two-electrode setup (using Agilent Fractionator 3100 and an optimized potential program) D) Two-electrode setup using standard method recommended by Agilent Technologies in their operator manual.

In order to underline the advantages of the multi-electrode setup, the separation of the same protein mixture (*i.e.* β-lactoglobulin, α-lactalbumin, cytochrome C, myoglobin and RNase A, 26 μg/mL each) was performed using a two-electrode setup. All the experimental conditions employed with the multi-electrode setup were also used for the OFFGEL electrophoresis with two electrodes (*e.g.* separation frame, length of the gel, amount and position of protein loaded, temperature) to have a reliable comparison between the multi-electrode and two-electrode setups. A part from the fact that the electric field was applied only between the two electrodes placed at both extremes of the multi-well frame, three different variations were tested regarding the potential program applied and the experimental conditions used (see Table 2.1).

Table 2.1. Experimental conditions employed during OFFGEL electrophoresis.

	Voltage, V	Current, µA	Power, W	Time, h
Multi-electrode setup	675	130[1]	-	3
Two-electrode setup	675	110[1]	200	3
Agilent fractionator 1	300 1000 5000	100[2]	200	3
Agilent fractionator 2	5000	100[2]	200	15

[1] Initial measured values
[2] Defined limiting current values.

Table 2.2. Table of common constituents in the protein sample and well number where the separated proteins were detected by MALDI-MS.

			Multi-electrode	Two-electrode	Agilent fractionator 1	Agilent fractionator 2	
Protein	pI	MW, kDa	No. well observed				No. well expected
α-lactalbumin	5.02	14,2	5,6(anodic side)	4,5	5,6	5,6(anodic side)	5,6
β-lactoglobulin	5.1	18,4	5	4,5	5	5,6	5
Myoglobin	7.0, 7.4	17	3	4	3,2	3,4	3
RNA A	9,45	13,6	1	1,2,4	1	1,2	1
Cytochrome C	9.6	12,2	1(cathode)	1,4	out	out	1

As shown in Table 2.2, for the two-electrode system all the proteins were observed in the well where they were added (well No. 4), which suggests that in these conditions the separation was not efficient. This result is in good agreement with the UV experiments, where 48% of all the added proteins were recovered from well No. 4.

After electrophoresis, the gel strip was stained using Coomassie Blue and the results showed the presence of a high amount of proteins inside the gel (see Figure 2.5). These results show that under the employed conditions (*e.g.* potential applied 675 V), an experimental time of 3 hours is not sufficient to properly separate all the proteins with a two-electrode setup. This is consistent with the numerical simulations, where a longer experimental time is needed to complete the fractionation of the same protein sample under the application of the same overall voltage.

The same protein sample and gel were used in a subsequent experiment using an Agilent Fractionator 3100 (*i.e.* two-electrode setup) and an optimized potential step program (see Table 2.1, Agilent fractionator 1). Under these conditions, cytochrome C was not observed, as it migrated beyond the limits of the well setup to the cathode. This is due to the position of the electrodes beside the extreme wells rather than inside them and the extreme p*I* of cytochrome C (p*I* = 9.6). However, a better protein separation was obtained thanks to the optimized potential program in comparison to the previous two-electrode experiment. Despite this, myoglobin was founded in two wells demonstrating that the separation efficiency achieved by using a two-electrode setup with a higher applied potential does not compete with that achieved by using the multi-electrode setup at a lower applied potential. The concentration of proteins after the separation was measured using UV-vis spectroscopy and summarized in Table 2.3. As it is clearly seen the collection efficiencies obtained after separation are quite low compared with the collection efficiencies in the case of the multi-electrode setup. A high amount of proteins were observed on the gel strip after the staining procedure.

Table 2.3. Protein recovery determined after OFFGEL separation using UV-spectroscopy.

No. well	Multi-electrode	Two-electrode	Agilent fractionator 1	Agilent fractionator 2
	Amount of protein recovery in µg/mL			
1	52µg/mL*	26 µg/mL*	19 µg/mL	22 µg/mL
2		10 µg/mL	2 µg/mL	4 µg/mL
3	26µg/mL		4 µg/mL	20µg/mL
4		62 µg/mL*		3 µg/mL
5	36 µg/mL*	20 µg/mL*	20 µg/mL*	33 µg/mL*
6	16 µg/mL		5 µg/mL	17 µg/mL*

- the value corresponds to the mixture of two or more proteins

Finally, an OFFGEL standard method (recommended by Agilent Technologies in the operator manual, Agilent fractionator 2 in Table 2.1 and 2.2) was selected to perform the protein separation of the same sample. The time employed for such experiments was 15 hours. However, β-lactoglobulin was still detected in several wells demonstrating not very effective resolution of the method. As in the previous experiment cytochrome C migrates out of multi-well device, due to the long experimental time employed. Analysis of the gel strip after the OFFGEL electrophoresis using a Coomassie Blue staining showed the presence of a high amount of proteins inside the gel. The concentrations of recovered proteins are higher in a comparison with that obtained under the method of Agilent fractionator 1, but still not as good as the one obtained with the multi-electrode setup.

3.3 OFFGEL fractionation of *E.coli* extract

To corroborate the capabilities of the multi-electrode setup for fractionation of complex protein samples, OFFGEL electrophoresis of an *E. coli* extract were performed with a multi-electrode and a two-electrode (Agilent OFFGEL fractionator) systems. The experimental conditions employed in both experiments were exactly the same (*e.g.* separation frame, length of the gel, temperature), except the experimental time and difference of potential applied in each case. The extract from *E.coli* was prepared in 0.5% IPG buffer with a protein concentration approximately of 40 μg/mL and added in each well of the multi-well device. The sample was spiked with

10μg/mL solution of cytochrome C in order to observe the effect of a complex matrix such as *E. coli* on the separation of this model protein. The potential program applied with the multi-electrode setup corresponds to the one shown previously in Table 2.1. In the case of the two-electrode setup, the standard method (Agilent fractionator 2) was used. After OFFGEL electrophoresis with both methodologies, soluble fractions from each well were collected and further separated by SDS-PAGE to obtain a complete two-dimensional map (*i.e.* 2D-PAGE) of the present proteins in the *E. coli* extract. The SDS-PAGE experiments were run for 2 hours with a starting applied potential of 60 V, after 1 hour the applied potential was increased up to 100 V. After SDS-PAGE, the gels were silver stained under the same conditions (*i.e.* same staining kit and protocol) to first visualize the separated proteins and second to compare the protein separation efficiency between the two employed methodologies. Figure 2.6 shows the results obtained after silver staining of the two electrophoretic separations, where each track corresponds to the separation of the proteins collected from each well. As it can be seen, the protein separation made with the multi-electrode setup shows a lower number of protein bands, but with a higher intensity. The latter is most likely due to a better separation and focusing of the proteins present in the *E. coli* extract. For instance in Figure 2.6A, a considerable protein population is observed in the region of proteins with high molecular weight and basic p*I*s, which is in disagreement with the literature [24] as there should not be a high amount of proteins with a basic p*I* values in the analyzed *E. coli* extract. The fact that the separation performed with the multi-electrode setup does not show such protein population (see Figure 2.6B) demonstrates that the present methodology can be applied to the analysis of complex samples avoiding the introduction of artifacts into the protein separations. Moreover, it can be seen that the spiked cytochrome C has been separated in both cases, but only with the multi-electrode setup a clear and intense protein band is visualized that corroborates the capabilities of the multi-electrode setup as a powerful tool for protein separation even in the presence of complex matrices. This result confirms that a higher protein separation and a higher protein collection efficiency is achieved by using the multi-electrode setup concept, which additionally can drastically shortened the experimental time required for a complete protein separation.

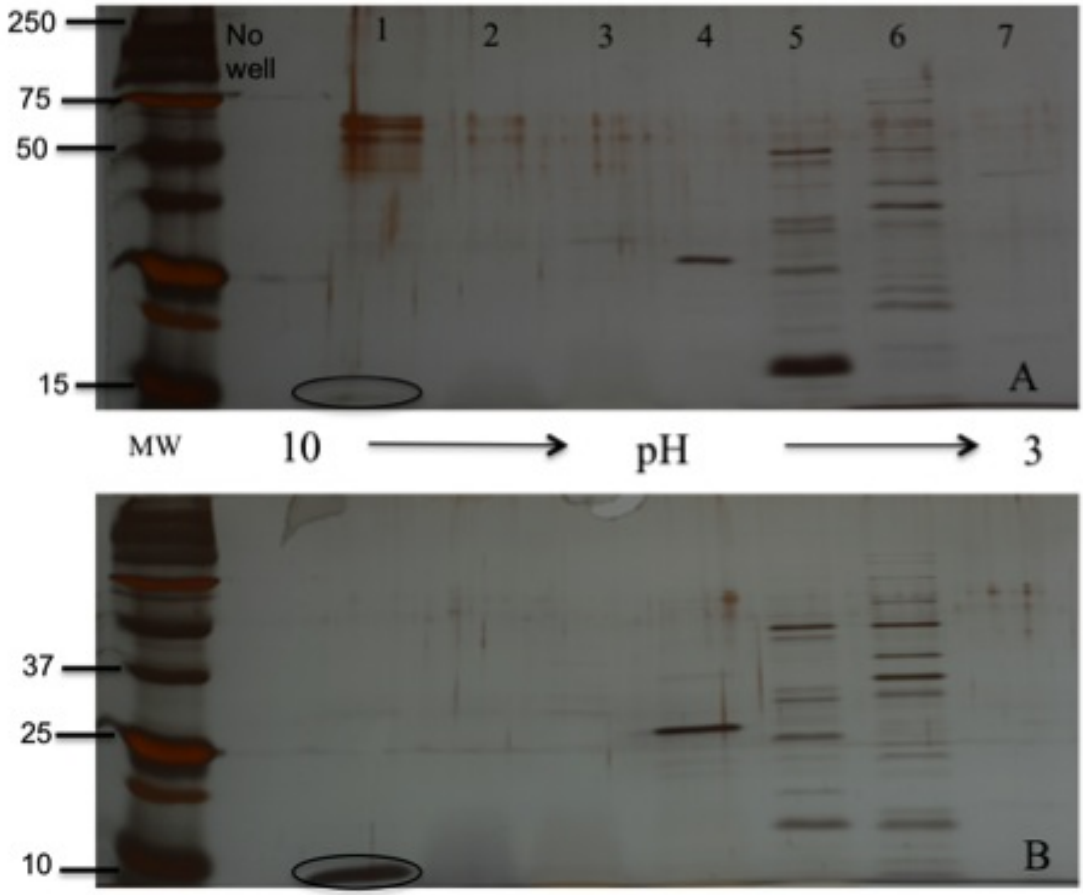

Figure 2.6. Silver Stained 2-D gels of an *E. coli* protein extract after fractionation on an IEF gel with a pH range between 3 and 10 A) by Agilent OFFGEL Fractionator (15h) and B) by a multi-electrode setup (3h). Each well of SDS-PAGE matches the protein fractions collected from a respective well after OFFGEL separation. Pre-stained Molecular weight protein markers appear on left side of the gel and are as follows from top to bottom: 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa.

4. Conclusions

A multi-electrode concept for improving OFFGEL electrophoresis based on the application of a more homogeneous electric field has been developed. Numerical simulations showed that the applied electric field with two-electrode devices is not uniform along the separation media (*e.g.* IPG gel) and therefore long separation times are required for a complete protein separation. In contrast, the multi-electrode setup provides a more uniform electric field offering faster and better protein IEF separations. To demonstrate experimentally this concept, OFFGEL electrophoresis were performed for the separation of ideal and complex samples with a multi-electrode setup and compared with usual two-electrode OFFGEL electrophoresis. These results confirm that not only higher protein separation, but also better protein collection efficiency is achieved in a shorter time by using the multi-electrode setup for OFFGEL experiments. We expect that the multi-electrode concept will shorten and refine proteome research based on gel electrophoresis.

Appendix 2.0

1. Physical constants:

The constants used in finite element simulations for numerical analysis are summarized in the table below.

Table A2.0. Constant parameters for the numerical simulations.

Constant	Value	Units
R (gas constant)	8.31	J/mol*K
T (temperature)	298	K
F (Faraday constant)	96485	C/mol
δ_{DI} (water conductivity)	$5.5*10^{-6}$	S/m
H^+ (diffusion coefficient of H^+)	$9*10^{-9}$	m^2/s
OH^-(diffusion coefficient of OH^-)	$5.27*10^{-9}$	m^2/s
D (diffusion coefficient of α-lactalbumin)	$1.06*10^{-10}$	m^2/s

pK_a values for amino acids were from the Handbook of Chemistry and Physics (http://www.hbcpnetbase.com/)

Due to numerical instabilities for the convergence of Nernst-Planck equation the overall potential difference was set to 1 V. The choice of the potential difference is arbitrary for comparison of the efficiency and separation rate of different electrophoretic setups and voltage programs as the only requirement is to maintain equal conditions for electrophoretic separations.

Table A2.1. The potential programs used in numerical simulations.

	Electrode No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Multi-electrode setup	Optimized potential program for general protein mixtures (V)	0	0,211	0,359	0,453	0,606	0,770	1
Multi-electrode setup	Adjusted voltage program for separation of proteins with acidic p*I* (V)	0	0,05	0,125	0,225	0,35	0,5	0,675
Two-electrode setup	Potential program used in two-electrode setup (V)	0	-	-	-	-	-	0,675

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Chapter III.

Proteins/peptides purification by three-well OFFGEL electrophoresis with immobilized ultra narrow pH gradient gels

Based on E. Tobolkina et al. Analytical Methods, 2014, 6(12), 3995-4002.

1. Introduction

Proteomics presents very demanding challenges for bioanalytical chemistry, due to the highly complex samples and the wide dynamic range of analyte concentrations. To date, the most widely used protocol is the bottom-up proteomics approach[1, 2], where proteins are digested and the generated peptides are separated by liquid chromatography (LC) and identified by tandem mass spectrometry (MS/MS)[3, 4]. Alternatively, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is used in top-down proteomic approaches[5], which includes a high resolution separation of proteins according to their p*I* in the first dimension by isoelectric focusing (IEF) and then according to their molecular weight (MW) in the second dimension after denaturation by sodium dodecylsulfate. The 2D-PAGE has a high capacity for the fractionation of complex mixtures of proteins, permitting to resolve hundreds or even thousands of sample spots[6-8].

IEF separates proteins or peptides according to their isoelectric points (p*I*) in a stable pH gradient under the application of an electric field [9]. The pH gradient was originally established with a mixture of carrier ampholytes. Nowadays, the most widely used pH gradient media are the immobilized pH gradient (IPG) gels where a series of ampholytes with specific p*I* values and high buffering powers are precisely organized along the gel to generate a linear pH gradient. IEF can also be performed in an OFFGEL manner[10] in a multi-well device[11], where the gel is placed under an array of wells that are filled with sample solutions. Proteins or peptides are fractionated according to their isoelectric points, while the separated components are directly recovered in liquid fractions on top of the gel and thus easily analysed by other techniques such as LC-MS[12-14]. In addition of being a high-resolution separation technique, IEF electrophoresis also provides information on the sample isoelectric point that is valuable for identification. For instance, it has been suggested that a confident identification of peptides can be obtained by employing both the information of molecular weight from MS and p*I* from IEF even without tandem MS[15].

Considering the complexity of proteomic samples, new simple strategies and methods are still needed to pre-fractionate, separate, detect and identify the relevant species[16] even if many techniques for preparative purposes, *e.g.* fractionation,

simplification and purification of tissue extracts, have already been developed [17-20]. The proteomic samples, depending on their nature, may contain a high concentration of salts or detergents, causing a major problem for mass spectrometry analysis. The use of microchip[21] and OFFGEL flow cells[22] were proposed in order to overcome the time-consuming desalting techniques, such as dialysis,[23] gel filtration[24] or precipitation techniques[25]. To decrease the experimental time and to improve the protein recovery, a multi-electrode setup was introduced recently for OFFGEL electrophoresis as described in Chapter II.

In this chapter, an electrophoretic separation method with an immobilized ultra narrow pH gradient (UNPG) gel centred on a mean pH value is studied. In comparison to the previous OFFGEL reports, the present device is a simplified preparative proteomic technique for fast sample purification and desalting. Proteins/peptides can be separated according to their electrophoretic mobility at the mean pH value and fractionated according to their charge at this pH. Immobilized UNPG gels can be obtained similarly to IPG gels by polymerization of acidic or basic monomers (Immobilines) in different ratios or simply obtained by cutting an area with a defined pH value from a commercially available IPG plate gel.

Using UNPG gels, ampholytes were rapidly fractionated by a three-well OFFGEL device into 3 fractions: the cathodic one containing proteins or peptides having a p*I* larger than the mean pH value of the gel in the reservoir where the cathode, the anodic one for species with a p*I* smaller than the mean pH of the gel and a third one in the middle reservoir for species that have a p*I* contained into the narrow pH range. This concept was first demonstrated by separating a mixture of four proteins followed with protein identification by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). The protein recovery was measured by UV-Vis spectroscopy. Additionally, purification of an *Escherichia coli* (*E. coli*) extract was performed, demonstrating that the proposed methodology can be applied to complex samples. Furthermore, an *E. coli* protein extract was digested and the peptide mixture was firstly separated in the three-well device and then analysed by LC-MS/MS analysis. Moreover, it was shown that the three-well device could be used for partial desalting. All the results suggested that electrophoretic separation with UNPG gels provides an efficient strategy for fast purification of protein mixtures, and can thereby be used as a preparative technique for proteomics.

2. Materials and methods

2.1 Materials

OFFGEL: Immobiline® DryPlate, linear pH range from 4.0 to 7.0 was purchased from Amersham Biosciences (Uppsala, Sweden). **Proteins and solutions:** α-casein (p*I* 4.6) from bovine milk was from Fluka (Buchs, Switzerland). α-lactalbumin from bovine milk type I (p*I* 5.02), β-lactoglobulin A from bovin milk (p*I* 5.1), myoglobin from horse skeletal muscle (p*I* 7.0, 7.4) and cytochrome C from horse heart (p*I* 9.6) were obtained from Sigma-Aldrich (Schnelldorf, Switzerland), as well as, sinapinic acid, acetonitrile, trifluoroacetic acid, methanol and acetic acid of the purest grade (>99.9 %). Deionized (DI) water was purified by an alpha Q Millipore system (Zug, Switzerland) and used in all aqueous solutions. **Digestion and LC-MS/MS:** 1,4-dithio-DL-threitol (DTT, > 99.5%), urea and iodoacetamide (IAA) were from Fluka (Buchs, Switzerland). Trypsin from bovine pancreas was from AppliChem (Darmstadt, Germany). **SDS:** sodium dodecyl sulfate (SDS), Trizma base, bromophenol blue, ammonium persulfate (APS), N,N,N',N'-tetramethylenediamine (TEMED), acrylamide and bis-acrylamide solution were all from Sigma (Buchs, Switzerland). Silver staining kit for protein visualization was purchased from Amersham Biosciences (Uppsala, Sweden).

2.2 UV-Vis spectroscopy

The UV-Visible absorption spectra were obtained with a standard spectrophotometer (Perkin Elmer, model Lambda XLS+) using quartz cells with a path length of 1 cm. A calibration bicinchoninic acid (BCA) protein assay kit for determining protein concentrations was obtained from Thermo Scientific (Rockford, USA).

2.3 Soluble E. coli protein extract preparation

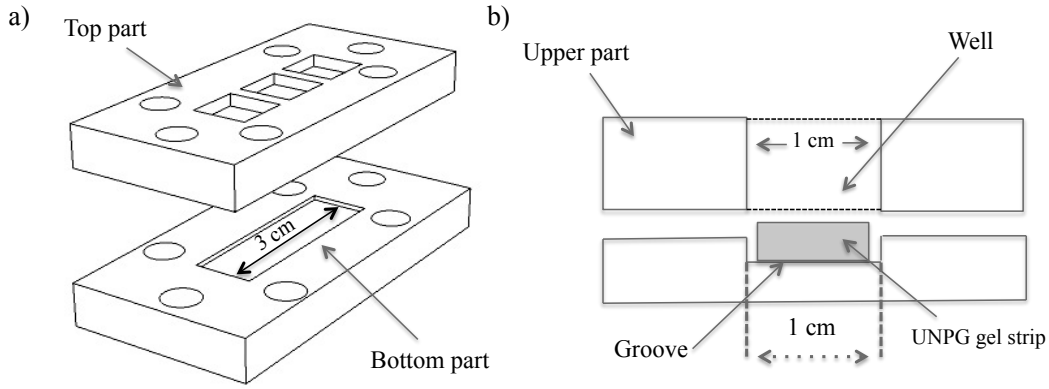
An over-night 200 mL culture of *E. coli* (strain DH5α) was collected by 10 min centrifugation at 5000 *g* and 4 °C. The cell pellet (0.6 g wet weight) was re-suspended in 3 mL of lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA) and cells were disrupted by sonication (10 x 10 pulses of 1 s at 30 W). Cell debris were removed by 10 min centrifugation at 2000 *g*. Ultracentrifugation (1 h at 100000 *g* at 4 °C) was applied to the total cell extract to remove membranes and membrane-bound proteins. The supernatant was collected and was considered as the soluble fraction of *E. coli* proteins.

2.4 Tryptic digestion

30 μL of Urea (18 M) was added to 70 μL of *E. coli* protein extract with the initial concentration of 10 mg/mL. 5 μL of 200 mM DTT in 50 mM Tris-HCl was added to the extract mixture and incubated for 1 h at room temperature in the dark. Afterwards, 20 μL of 200 mM IAA in 50 mM Tris-HCl was added and the mixture was incubated for 1 h at room temperature in the dark. To consume any unreacted iodoacetamide, 20 μL of 200 mM DTT in 50 mM Tris-HCl was added. To reduce the urea concentration to ≈ 0.6 M, 775 μL of 50 mM Tris-HCl solution was added. Finally, the trypsin was added to the solution with a final trypsin:protein ratio of 1:50 (w/w). The digestion was performed at 37 °C overnight.

2.5 Electrophoretic purification with immobilized UNPG gels

A commercial gel plate with a linear pH range 4 – 7 (Immobiline DryPlate pH = 4 – 7, T = 4%, C = 3%) was cut at the desired mean pH value to obtain gel strips with an ultra narrow pH gradient ≈ 0.27 pH units per cm. After re-swelling in water for 1 hour, the strip was inserted in a simple three-well OFFGEL device (see Scheme 3.1) for separating the ampholytes according to their charge at the chosen pH and also for sample desalting.



Scheme 3.1. Schematic representation of a) the top and bottom view of the three-well device, and b) cross-section view of the three-well device with an UNPG strip placed in the groove of the bottom part.

During electrophoresis, Pt electrodes were placed at the outer wells. Each of the wells was filled with 150 μL of DI water. The sample was loaded in the middle well and electrophoresis was performed with a high voltage power supply of Pharmacia Biotech EPS 3500 XL (Sweden). It is very important to keep the sample in a concentration range equal to 1 – 10 mg/component/cm of gel in order to avoid

overloading of the sample and possible electroosmotic flow [9]. The purification was performed either with 10 μL of a protein mixture, containing α-casein, α-lactalbumin, cytochrome C and myoglobin (26 μg/mL each) or with 30 μL of *E. coli* protein or its digest. Three-well OFFGEL electrophoresis in all the cases was performed under the following conditions: 30 min at 150 V and then 4-5 h at 400 V, the current limit was set at 1 mA. After fractionation with the immobilized UNPG gels, soluble fractions were collected from all the wells and further separated by MS analysis or by SDS-PAGE to obtain a two-dimensional map of the proteins present in the *E. coli* extract. SDS-PAGE was always run by 2 hours with a starting applied potential of 60 V and 100 V after 1 hour.

2.6 MALDI-MS analysis

The protein analyses were performed on a Microflex LRF MALDI-TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with a 337 nm nitrogen laser. 1 µL of the extracted protein fraction from the three-well device was deposited on a steel target plate to dry under ambient conditions. Afterwards, 1 μL of sinapinic acid (SA) matrix (15 mg/mL sinapinic acid in 50 % acetonitrile, 0.1 % trifluoroacetic acid and 49.9 % water) was added and left to dry at room temperature and pressure.

2.7 LC-MS/MS analysis

Liquid chromatography was performed on an ACCELA LC system (ThermoFisher Scientific, Reinach, Switzerland). 20 μL of *E. coli* digest was loaded on a C-18 reversed-phase packed column (2.0 mm ID × 150 mm, COSMOSIL, $5C_{18}$-MS-II) with a loop loading speed of 8.0 μL/s. The mobile phase consisted of A (ACN/TFA 99.95/0.05 (v/v)) and B (water/TFA 99.95/0.05 (v/v)). Gradient was from 5 to 50 % of solvent A in 66 min, followed by the increase from 50 to 95 % of A in 29 min. The mobile phase flow rate was always 200 μL/min. Electrospray ionization tandem MS (ESI-MS/MS) analysis were performed on a linear ion trap mass spectrometer of Thermo LTQ Velos (ThermoFisher Scientific, Reinach, Switzerland). MS/MS detection was operated in a positive ion mode with *m/z* 400-2000 scanning range and collision induced dissociation. LC-MS/MS data processing was performed with the Trans-Proteomic Pipeline (TPP), which is a set of integrated tools for MS/MS proteomics, developed at the Seattle Proteome Center of the Institute for Systems Biology in USA (http://tools.proteomecenter.org/wiki/index.php?title=Software:TPP). The peptide

assignment was performed with X! Tandem as search engine and UniProtKB as database. Results validation was performed with the PeptideProphet of TPP.

2.8 Simulations

The simulation of electrical properties of the three-well device was performed using commercial COMSOL Multiphysics (version 3.5a), employing finite element method, operating under Ubuntu 8.04 environment installed on a MacPro with four core 2.66 GHz processing units and 9.8 Gb of RAM. Poisson equation (eq. 3.1) was solved in two-dimensional computational domain of the three-well setup.

$$\nabla^2\phi = -\frac{F}{\varepsilon_0\varepsilon_r}\sum_i z_i c_i \qquad (3.1)$$

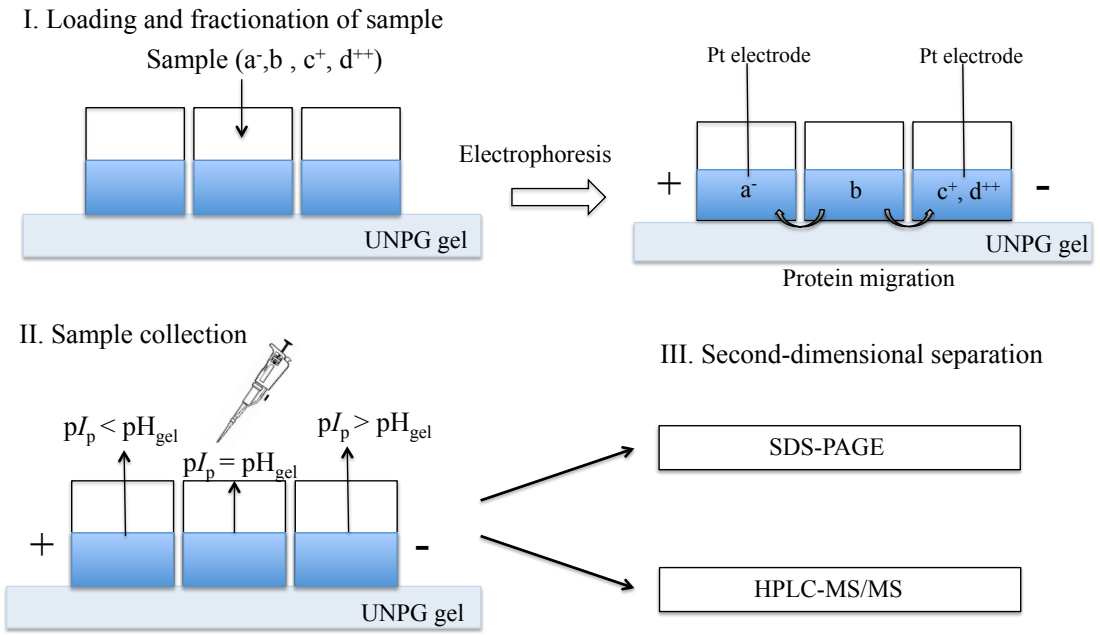
Here ϕ, c_i, z_i, F, ε_0, and ε_r, denote electrical potential, the concentration, the charge number of species i, the Faraday's constant, vacuum and relative permittivity, respectively.

Numerical resolution of the Poisson equation system was computed using 7946 mesh elements corresponding to 15295 degrees of freedom. The model boundaries (except the wells where the electrodes were placed) were set to insulation conditions, a potential difference of 1 V was set between subdomain 1 and 3.

3. Results and discussion

3.1 Principle: electrophoretic separation with immobilized UNPG gels

The electrophoretic separation/desalting method using a polyacrylamide UNPG gel is based on the electrophoretic mobility difference of charged proteins or peptides loaded in the central well of the device as illustrated in Scheme 3.2.



Scheme 3.2. Schematic representation of an UNPG gel electrophoretic separation method coupled with a second-dimension separation of HPLC-MS/MS or SDS-PAGE. pH_{gel}: the mean pH value of the UNPG gel; pI_p: the isoelectric point of proteins or peptides.

Upon application of an electric field, the proteins/peptides migrate in and over the polyacrylamide gel having a defined mean pH value. The proteins/peptides with p*I* higher than the mean pH of the gel will be positively charged (c^+, d^{++} in Scheme 3.2) and therefore will migrate towards the cathode. Additionally, the proteins with larger net charge (d^{++}) or the smaller size will migrate faster than the others allowing a protein fractionation based on mobility. In contrast, the proteins/peptides (a^-) with a p*I* lower than the mean pH of the gel will be negatively charged and therefore migrate towards the anode. The proteins/peptides (b) with a p*I* equal or similar to the mean pH of the gel are globally neutral and then stay in the middle well without significant migration. Concentration gradient and diffusion can happen in the first and third well but not in the middle well because of isoelectric focusing. In addition to sample fractionation, the UNPG gel electrophoresis can also be used for sample desalting

when the target proteins/peptides have a p*I* close to the mean pH of the gel, where samples will stay in the middle well and salts will migrate to the cathode or anode.

An important parameter for this methodology is the migration velocity (V_m) of the ampholytes determined both by their charge and their electrochemical mobility. V_m is classically defined as (eq. 3.2):

$$V_m = -\frac{zF}{RT} D\nabla\phi = -zF\tilde{u}\nabla\phi = uE \qquad (3.2)$$

where z is the charge of the ampholyte at the mean pH value, F is the Faraday constant, R is the gas constant, T is the temperature, D is the diffusion coefficient of the ampholyte, $\tilde{u}$ is electrochemical mobility, u the electrophoretic mobility and E is the applied electric field [26].

Indeed, the migration of charged species during an electrophoretic separation follows the electric field according to the Nernst-Planck equation. In the present case, the separation is conducted in and above the gel in a three-well configuration. Figure 3.1 illustrates the distribution of electric field (E_n) demonstrating a significant inhomogeneity, especially near the well edges (see also a cross-section view on E_n distribution on Figure 3.1b). Even in the absence of an electrode in the central well where the sample is loaded, the electric field penetrates the liquid medium and further drives charged species into the gel, where the separation occurs according to the pH of the gel.

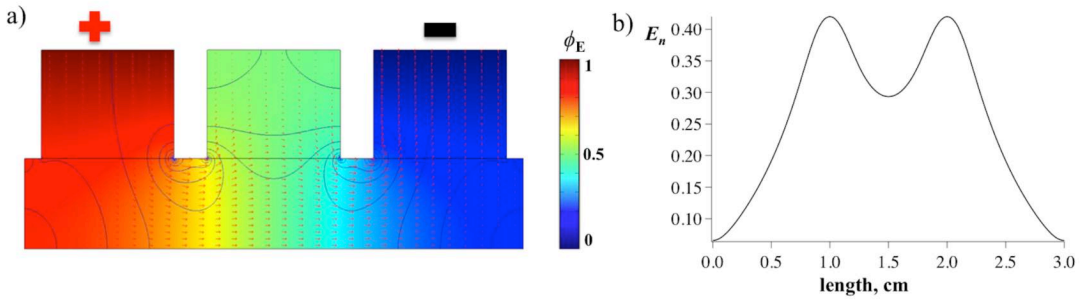


Figure 3.1. Simulated electrical properties in a three-well device. a) The overlay of color map illustrating electric potential distribution (ϕ_E) and contour plot depicting the isovalue lines for electric field (E_n) and b) the distribution of electric field along the central cross-section inside the UNPG gel. For simplicity, the electrodes are considered to be at the top surface of the outer reservoirs.

3.2 Purification on UNPG gels

Protein purification can be realized according to their charge with a very short UNPG gel strip. A mixture of four proteins with different p*I* values (α-casein (p*I* = 4.6), α-lactalbumin (p*I* = 5.02), cytochrome C (p*I* = 9.6) and myoglobin (p*I* = 7.0), 26 µg/mL each) was loaded in the middle well and separated on a UNPG gel (pH = 5-5.27). The applied potential difference between the electrodes was 150 V for 30 min and then increased up to 300 V for 3 h, the limiting current was 1 mA. Due to the fact that cytochrome C and myoglobin are coloured proteins, it was easy to visualize their migration inside the gel. At the end of the separation, the fractions were collected and analysed by MALDI-MS (see Figure 3.2). UV-Vis spectroscopy was employed to quantify the amount of proteins collected after fractionation. The results are summarized in Table 3.1.

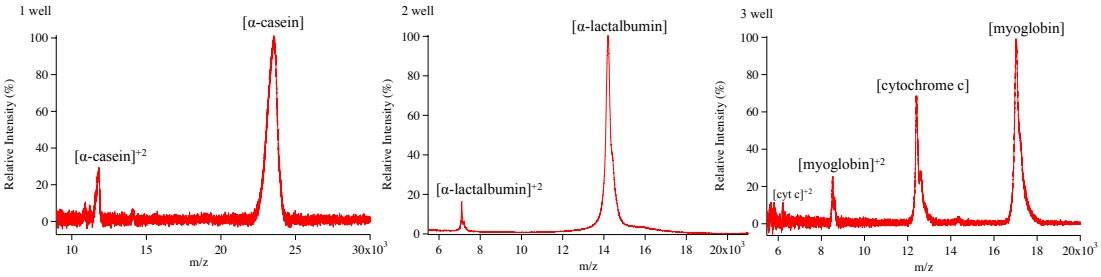


Figure 3.2. Mass spectra of the fractions from wells No. 1 (anodic side), 2 (middle well) and 3 (cathodic side) after electrophoresis performed with the three-well device over an UNPG gel (pH = 5-5.27). The samples were analysed by MALDI-MS with sinapinic acid matrix.

According to the MS results shown in Figure 3.2 and the UV-Vis quantitative analysis summarized in table 3.1, all the proteins were completely fractionated according to their p*I* and reached the expected position (*i.e.* wells). For instance, α-lactalbumin was detected from the middle well, since the p*I* of this protein is within the pH range of the UNPG gel. Cytochrome C and myoglobin migrated to the third well (cathodic side), due to the fact that they were both positively charged. Similarly, the negatively charged α-casein migrated to the first well (anodic side). The protein recovery after separation was analysed by UV-Vis spectroscopy showing very high recovery in the central well used for purification. The recovery yields in the side wells are less due to the sample loss in the polyacrylamide gel and perhaps to the reaction occurring on the electrode. As depicted in Figure 3.1, the electric field near the well

edges is low, which can lead to not efficient protein recovery. In order to increase the protein recovery in wells 1 and 3 longer experimental time is required.

This relatively high recovery resulted from the large solution to gel volume ratio; therefore increasing the amount of solution or decreasing the size of gel might lead to even higher protein recoveries. However, an increased solution-to-gel volume ratio can also lead to decreased buffer capacity of the system, and optimized ratio should be considered for both sample separation and recovery.

Table 3.1. Protein recovery determined after electrophoretic separation in the three-well device over an UNPG gel (pH 5-5.27) using UV-spectroscopy.

Well No.	Identified proteins	Initial concentration of the proteins loaded	Amount of protein recovery	Protein recovery, %
1 (anodic side)	α-casein	26 μg/mL	13 μg/mL	62%
2 (middle)	α-lactalbumin	26 μg/mL	25 μg/mL	96%
3 (cathodic side)	myoglobin, cytochrome C	52 μg/mL*	35 μg/mL	67%

* This value corresponds to the summed concentration of the two present proteins.

3.3 Sample desalting on UNPG gels

In addition to separation, protein/peptide samples can also be rapidly and easily desalted using the three-well device on an UNPG gel. A gel strip with a pH gradient in the range of 5-5.27 was introduced into the device and 100 μL of DI water was added into each well. 10 μL of the solution of β-lactoglobulin A (1 mg/mL, pI = 5.1) containing 1 M NaCl was loaded into the middle well and the following experimental conditions were employed for the sample desalting: voltage applied = 100 V, limiting current = 1 mA. The pH of the gel was chosen to fit the requirements of the electrophoretic separation principle (see section III.3.1) that means the proteins with pI equal or similar to the mean pH of the gel would not migrate from the well where they were loaded. After the electric field was applied along the three-well system, the Na^+ and Cl^- ions penetrated inside the gel and started to migrate to the electrodes with

an opposite charge, while redox reaction happened on the electrodes. In 1 hour the fractions from each well were collected and analysed by MALDI-MS (see Figure 3.3). Since no protein was detected in the well No. 1 and 3, only the mass spectrum of the middle well, containing the *β*-lactoglobulin A is shown.

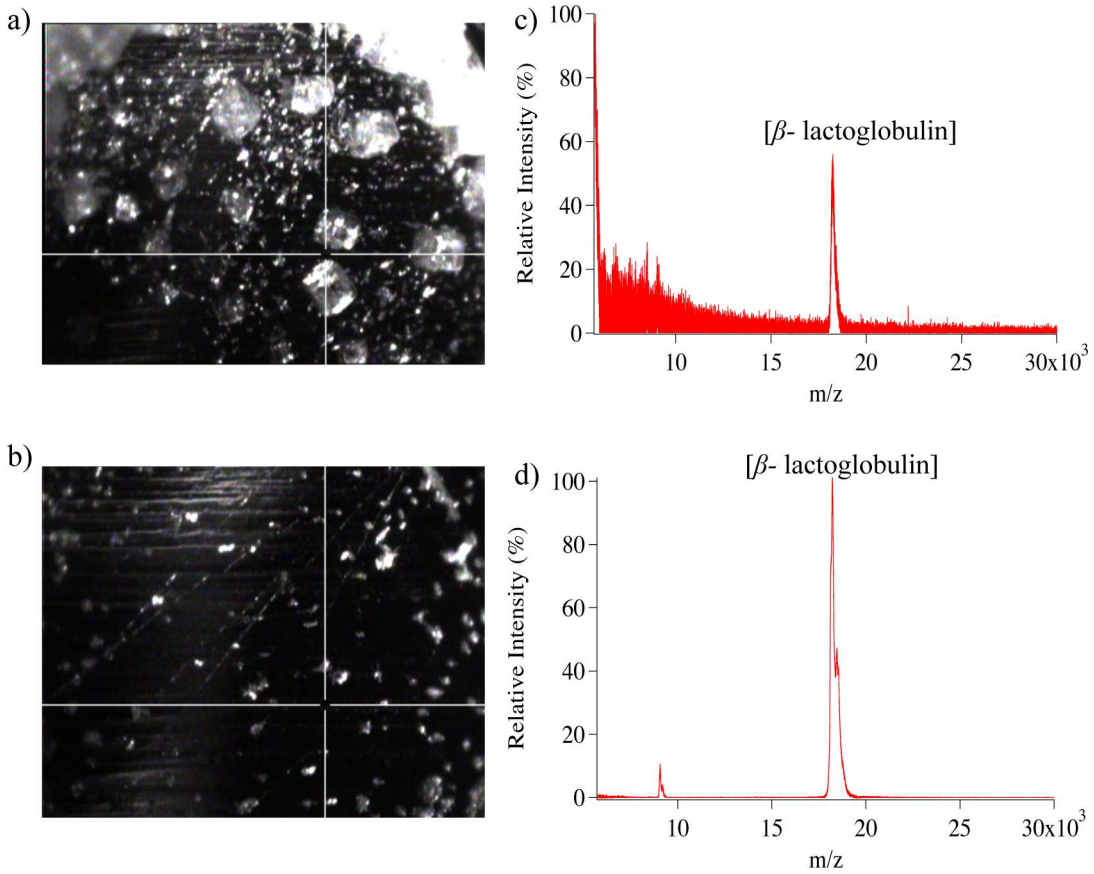


Figure 3.3. Optical image of the dried sample (1 mg/mL *β*-lactoglobulin A with 1 M NaCl) crystal on MALDI plate a) before and b) after the desalting by the three-well device with an UNPG gel (pH 5-5.27) and the obtained mass spectra of *β*-lactoglobulin A c) before and d) after the desalting. The samples were analyzed by MALDI-MS with sinapinic acid matrix.

Figure 3.3 illustrates the desalting effect of the three-well device using the UNPG gel. Figure 3.3a and 3.3b show the MALDI plate image containing the protein sample with 1 M NaCl before and after the desalting. As it can be observed the crystals of sample after the desalting are smaller in comparison to those ones observed before desalting, suggesting the removal of NaCl. The desalting effect is also demonstrated by the mass spectra of *β*-lactoglobulin A. Figure 3.3d presents a much better MS signal for the protein in comparison to the one in Figure 3.3c. Indeed, the laser intensity was increased by 20% to get the mass spectrum in Figure 3.3c compared to that in Figure 3.3d. These results show that the three-well device with an UNPG gel can provide effective and rapid desalting prior to further analysis.

3.4 Purification of E. coli extract on UNPG gels

To determine the capability of purifying complex samples and to show that the electrophoretic separation with UNPG gels could be used as a fractionation strategy prior to other separation techniques, such as SDS-PAGE, a three-well fractionation of an *E. coli* protein extract spiked with α-casein and cytochrome C was performed using a gel with a pH gradient of 5-5.27. The extract from *E. coli* was prepared with a protein concentration approximately of 30 μg/mL and added in each well of the device. The sample was spiked with cytochrome C and α-casein (3 μg/mL each) in order to observe the effect of such a complex matrix on the proteins fractionation. The fractions collected after fractionation in a three-well device were further separated by SDS-PAGE electrophoresis. The gels were silver stained to visualize the separated proteins (see Figure 3.4). SDS-PAGE of the pure *E. coli* extract presents a high degree of protein band overlapping, which is raised from the complexity of this protein mixture. After the fractionation in a three-well device using the ultra narrow pH gradient gel, the spiked α-casein (pI = 4.6) and cytochrome C (pI = 9.6) were separated according to the charges developed at chosen pH range. Indeed, clear and intense protein bands were visualized and detected in the expected positions (*i.e.* wells) that corroborates the capabilities of the three-well setup as a powerful tool for protein purification even in the presence of complex matrices. The fraction taken from the middle well with the pH close to 5.13 shows the highest protein population. Moreover, a well-resolved SDS-PAGE for each well has been obtained compared to the whole *E. coli* extract. Thus, it confirms that the electrophoretic separation in UNPG gels provides fast and efficient fractionation of proteins and can be applied to the analyses of complex samples.

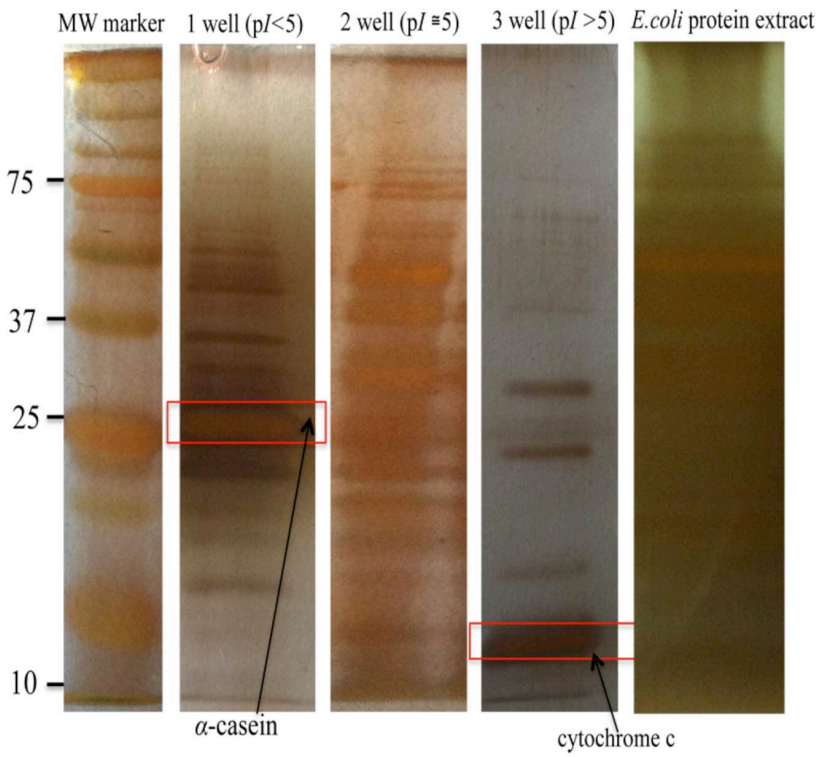


Figure 3.4. a) Silver stained SDS PAGE of *E. coli* protein extract spiked with α-casein and cytochrome C after fractionation on a UNPG gel with a pH gradient = 5.0-5.27. Each SDS-PAGE matches the protein fractions collected from a respective well after OFFGEL separation. Pre-stained Molecular weight protein markers appear on left side of the gel and are as follows from top to bottom: 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa.

3.5 Purification of digested E. coli extract followed by LC-MS/MS analysis

The purification of peptides derived from the tryptic digest of an *E. coli* protein extract was also carried out on an UNPG gel. An aliquot of the protein digest (30 μL) was loaded in the middle well of the three-well device on a gel strip with a pH gradient (pH= 6-6.27, mean pH=6.15). Then, 200 μL of water solution was added on top of each well. During the fractionation, the potential increased from 150 to 400 V in 6 hours with a limiting current of 1 mA. After electrophoresis, the peptide mixture was separated into three fractions, which were present in the liquid phase and can be easily collected for further analysis, for instance, by LC-MS/MS analysis. Each fraction was firstly lyophilized and re-dissolved in 30 μL water, then injected into the LC for LC-MS/MS analysis. The LC-MS/MS data of each fraction were searched against *Escherichia coli* database from UniProtKB by X! Tandem search engine and validated by Trans-Proteomic Pipeline (TPP) tools. The identified peptides from each

well by LC-MS/MS are listed in Appendix. The threshold of probability values from TPP was varied for different LC-MS/MS data while the error rate was constant as 5%.

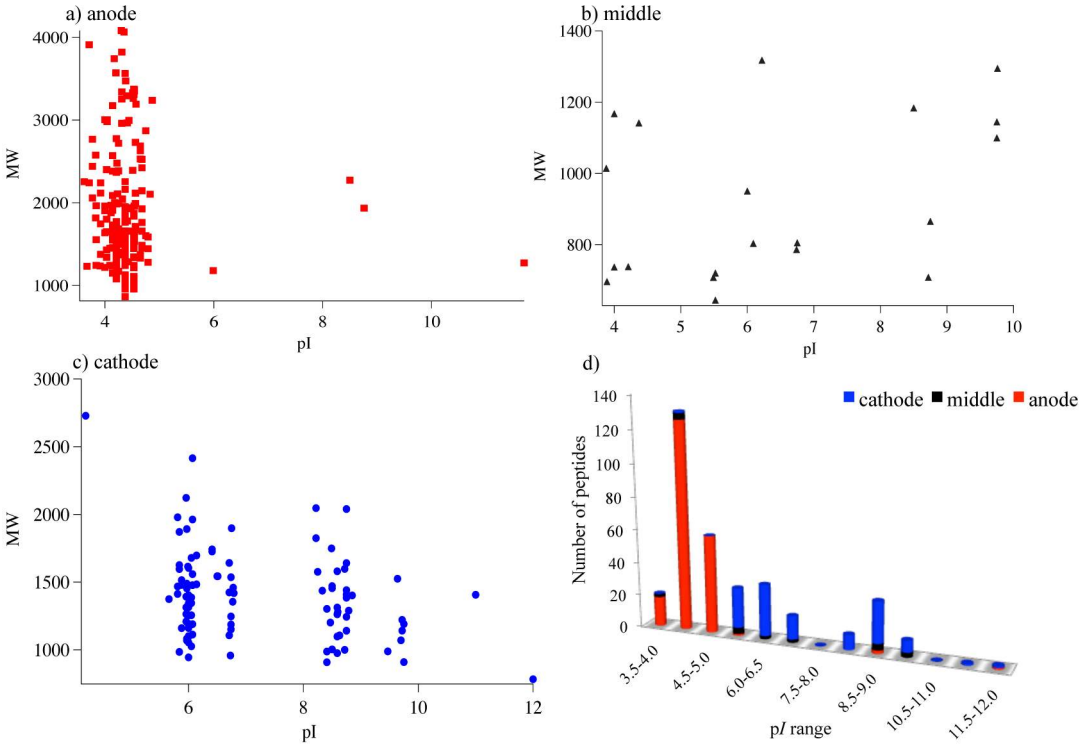


Figure 3.5. (a, b, c) Isoelectric points plotted as a function of molecular weights for the peptides identified from different wells. (d). Number of identified peptides after OFFGEL fractionation and LC-MS/MS analysis and their isoelectric point distribution.

In Figure 3.5, the p*I* values of the identified peptides from different fractions are compared. From the 1st well, 206 peptides were identified, where only 3 of them had a p*I* greater than 6. Indeed, the probability scores for these 3 peptides calculated by TPP are quite low, (*i.e.* 0.4233, 0.5485, 0.5839), and among the lowest ones, indicating that these 3 peptides are highly possible to be false positive identifications. 21 peptides were identified from the fraction of the middle well, where the sample was initially loaded. As shown in Figure 3.5, the p*I* of these 21 peptides is randomly distributed, indicating that a small fraction of the loaded peptides could not migrate into other wells. 113 peptides were identified from the third well by LC-MS/MS. Only one peptide does not match the p*I* range and actually with a low probability value from TPP as 0.735. In total 322 peptides with the correct p*I* were identified by LC-MS/MS after pre-fractionation step (see Appendix 3.0).

All in all, the present results demonstrate that the purification in the three-well device is efficient, and can be easily applied in the analysis of complex samples. Indeed, by optimizing the length of the UNPG-pH strip and its pH, specific and general protein (or peptide) sample fractionations can be obtained.

4. Conclusions

Protein fractionation by OFFGEL electrophoresis with an immobilized ultra narrow pH gradient gel in a three-well format has been presented. In comparison to previous OFFGEL electrophoresis reports[11], the present work aims at developing a fast and efficient preparative technique for isolation of proteins with specific p*I* from complex biological mixtures in a miniaturized format. The latter opens the possibility to use this preparative technique in combination with a following separation method to provide a powerful tool for proteomics. By tuning the mean pH of the UNPG gel, a specific target protein can be purified from a mixture of proteins in a short period of time. Fractionation in the three-well device drastically decreases the experimental time: it takes only 4 h to fractionate a complex mixture such as *E.coli* extract, while the normal OFFGEL electrophoresis takes up to 8 h. Furthermore, the pH gradient in UNPG gels is very low (0.27 units) and close to a constant pH media. To date, only few studies made by Stoyanov *et al.* [27, 28] can be found in the literature related to the use of uniform pH gels for protein fractionation. The proposed approach is low cost, easy to use and can be prepared in any laboratory.

Appendix 3.0

Purification of digested *E.coli* extract digest followed by LC-MS/MS analysis

Result obtained from the fraction No.1 (anodic side).

probability	peptide	protein	calc_neutral_pep_m	pI
1	K.C[160.03]DM[147.04]VDDEELLELVEM[147.04]EVR.E	sp|P0CE47	2254.9647	3.62
0.9993	K.AFQELNAIDVL.-	sp|P0AB71	1231.6447	3.67
1	K.ADEQILDIGDASAQELAEILK.N	sp|P0A799	2241.1377	3.71
0.8432	K.SGM[147.04]NPFLLDSEDVFIDLLTDSGTGAVTQSM[14	sp|P0A853	3911.7577	3.71
0.9997	K.DQIIEAVAAM[147.04]SVM[147.04]DVVELISAM[147	sp|P0A7K2	2768.3167	3.77
1	K.VEVETPEENTGDVIGDLSR.R	sp|P0A6M8	2057.9757	3.77
1	K.VALQDAGLSVSDIDDVILVGGQTR.M	sp|P0A6Y8	2440.2807	3.77
0.9997	R.TTDVTGTIELPEGVEM[147.04]VM[147.04]PGDNIK.M	sp|P0CE47	2577.2187	3.83
0.9993	R.TTDVTGTIELPEGVEM[147.04]VM[147.04]PGDNIK.M	sp|P0CE47	2577.2187	3.83
0.9999	K.VGDTVIEFDLPLLEEK.A	sp|P69783	1815.9507	3.83
1	R.VDDLAVDLVER.F	sp|P09373	1242.6457	3.84
1	K.DDSFFDVYTEC[160.03]R.T	sp|P0A853	1552.6137	3.84
1	R.ELLSQYDFPGDDTPIVR.G	sp|P0CE47	1963.9527	3.84
0.9999	K.EGQNLDFVGGAE.-	sp|P0ADZ0	1234.5467	3.91
1	R.SGETEDATIADLAVGTAAGQIK.T	sp|P0A6P9	2117.0487	3.92
0.9937	R.EM[147.04]LIADGIDPNELLNSLAAVK.S	sp|P0ACF8	2241.1567	3.92
1	K.AAGAELVGM[147.04]EDLADQIK.K	sp|P0A7L0	1745.8507	3.92
1	R.AFDQIDNAPEEK.A	sp|P0CE47	1375.6257	3.92
1	K.TLAEGQNVEFEIQDGQK.G	sp|P0A9Y6	1904.9117	4
0.9999	K.GM[147.04]NTAVGDEGGYAPNLGSNAEALAVIAEAVK	sp|P0A6P9	3004.4447	4
0.9999	K.VLYEM[147.04]DGVPEELAR.E	sp|P0ADY7	1635.7817	4
0.9966	K.ALEGDAEWEAK.I	sp|P0CE47	1217.5567	4
0.9945	K.ILELAGFLDSYIPEPER.A	sp|P0CE47	1961.0147	4
1	R.DIADAVTAAGVEVAK.S	sp|P0A7R1	1428.7457	4.03
1	K.ANDAAGDGTTTATVLAQAIITEGLK.A	sp|P0A6F5	2401.2337	4.03
0.9999	K.GGDTVTLNETDLTQIPK.V	sp|P0AET2	1800.9107	4.03
0.9992	R.AGDNAPM[147.04]AYIELVDR.S	sp|P0AG44	1649.7717	4.03
1	K.LINDAYDSEYFATK.V	sp|P0A6F3	1648.7617	4.03
0.9911	K.DGVIADFFVTEK.M	sp|P0A9X4	1339.6657	4.03
1	K.DKPEDAVLDVQGIATVTPAIVQAC[160.03]TQDK.Q	sp|P0AES9	2981.5017	4.04
1	K.DKPEDAVLDVQGIATVTPAIVQAC[160.03]TQDK.Q	sp|P0AES9	2981.5017	4.04
0.9993	K.DAM[147.04]VPM[147.04]GGLLC[160.03]M[147.04	sp|P0A853	3004.2417	4.04
1	K.ALEEAGAEVEVK.-	sp|P0A7K2	1243.6297	4.09
1	K.YLGGEELTEAEIK.G	sp|P0A6M8	1450.7187	4.09
0.9999	K.LKDGEDPGYTLYDLSER.L	sp|P69908	1969.9267	4.11
0.8707	K.LKDGEDPGYTLYDLSER.L	sp|P69908	1969.9267	4.11
0.9992	R.EQIIFPEIDYDKVDR.V	sp|P62399	1878.9367	4.11
0.9913	R.MDAAQLTEEGYYSVFGK.S	sp|P36683	1907.8607	4.14
1	R.LPGLYYIETDSTGER.T	sp|P37647	1712.8257	4.14
1	K.AAAFEGELIPASQIDR.L	sp|P0A7J3|	1686.8577	4.14
0.2873	R.NVEEGGSLTIIATALIDTGSK.M	sp|P0AG30	2088.0947	4.14
0.9848	K.IQTIDEIQSTETLIVLQNPIM[147.04]R.T	sp|P0ACI6|	2570.3627	4.14
1	R.NIGEILELAGC[160.03]DR.L	sp|P0A870	1458.7137	4.14
1	K.YDEAPSNVAQAVIEAR.G	sp|P0A6M8	1731.8427	4.14
1	K.GATVELADGVEGYLR.A	sp|P0AG67	1548.7787	4.14
1	K.EGEATLAPSLDLVGKI.-	sp|P0AE08	1611.8717	4.14
1	R.ATFVVDPQGIIQAIEVTAEGIGR.D	sp|P0AE08	2383.2747	4.14
0.3578	K.GNFDLEGLER.G	sp|P0A853	1148.5457	4.14
1	K.DIALGEEFVNK.-	sp|P61889	1233.6237	4.14
0.2801	R.TIAMSLPEFEQSLFMAAQPDNLLLATAPR.Y	sp|P31463	3174.6097	4.14
1	R.ATFVVDPQGIIQAIEVTAEGIGR.D	sp|P0AE08	2383.2747	4.14

1	K.VAEAIAASFGSFADFK.A	sp|P0AGD3	1629.8037	4.37
0.9989	K.TIATENAPAAIGPYVQGVDLGNM[147.04]IITSGQIPV	sp|P0AF93	3564.8497	4.37
0.999	K.QYDINEAIALLK.E	sp|P0A7L0	1389.7507	4.37
1	K.ALAINLVDPAAAGTVIEK.A	sp|P0AEE5	1764.9987	4.37
1	R.GWQVPAFTLGGEATDIVVM[147.04]R.I	sp|P69908	2162.0827	4.37
1	K.DLVESAPAALK.E	sp|P0A7K2	1112.6077	4.37
1	R.AQYLIDQLLAEAR.K	sp|P0AFG8	1502.8097	4.37
1	K.LVTDELVIALVK.E	sp|P69441	1311.8017	4.37
1	K.GLTDAAQQVVAAVEGK.-	sp|P0A836	1555.8207	4.37
0.9997	R.GLSVLM[147.04]LEAQDLAC[160.03]ATSSASSK.L	sp|P13035	2254.0817	4.37
0.9956	R.VTITIAADSIETAVK.S	sp|P0A850	1530.8507	4.37
0.9989	K.IVDLLTER.A	sp|P0A7D4	957.5497	4.37
1	K.AILAAAGIAEDVK.I	sp|P0A7S9	1240.7027	4.37
0.9925	R.YWDVELR.E	sp|P69908	979.4767	4.37
0.9991	K.TQLIDVIAEK.A	sp|P0ACF0	1128.6387	4.37
0.5307	K.ISDIPEFVR.G	sp|P0AC38	1074.5707	4.37
1	K.EVVFASIDTGTPAK.F	sp|Q46938	1433.7397	4.37
0.5328	K.TLQQLNASIAVEGLDAK.K	sp|P33362	1769.9527	4.37
1	K.LVADSITSQLER.R	sp|P0A7V3	1330.7097	4.37
1	R.IINEPTAAALAYGLDK.G	sp|P0A6Y8	1658.8877	4.37
0.9645	K.QLGEDPWVAIAK.R	sp|P0AG67	1325.6977	4.37
1	K.EGDAVQLVGFGTFK.V	sp|P0ACF0	1466.7407	4.37
0.9795	K.GQLKEFLDANLA.-	sp|P0AA25	1317.6927	4.37
1	R.DLALIEINPLVITK.Q	sp|P0A836	1550.9287	4.37
1	K.VQNASYQVAAYLADEIAK.L	sp|P69908	1952.9847	4.37
0.9984	K.VQNASYQVAAYLADEIAK.L	sp|P69908	1952.9847	4.37
0.9184	K.LGDIEYR.E	sp|P68066	864.4337	4.37
1	K.VVADIAGVPAQINIAEVR.K	sp|P0A7V3	1834.0317	4.37
0.972	R.TSTFLDVYIER.D	sp|P09373	1342.6767	4.37
0.6786	K.VYAAIEAGDK.A	sp|P0A7U7	1035.5237	4.37
0.9894	K.ELANVQDLTVR.G	sp|P02925	1256.6727	4.37
0.9998	K.VAIANVLKEEGFIEDFKVEGDTKPELELTLK.Y	sp|P0A7W7	3473.8547	4.38
0.9999	R.EAEGSHIM[147.04]LGAQNVDLNLSGAFTGETSAAM	sp|P0A858	3293.5547	4.4
0.9995	R.EAEYKDWTIEQITR.E	sp|P0A853	1780.8627	4.41
0.9989	K.AALESTLAAITESLKEGDAVQLVGFGTFK.V	sp|P0ACF0	2965.5647	4.41
0.9996	K.EYDHIKDVNDLPELLK.A	sp|P0A7A9	1939.9887	4.43
1	K.IALESVLLGDKE.-	sp|P0ABP8	1285.7127	4.43
1	R.GVVVAIDKDVVLVDAGLKSESAIPAEQFK.N	sp|P0AG67	2996.6437	4.44
1	K.AFTSEEFTHFLEELTK.Q	sp|P0A6P9	1927.9207	4.48
1	K.VGEEVEIVGIKETQK.S	sp|P0CE47	1656.8937	4.49
0.4239	R.VGFFNPIASEKEEGTRLDLDR.I	sp|P0A7T3	2392.2027	4.51
0.9999	R.VLENAEGDRTTPSIIAYTQDGETLVGQPAKR.Q	sp|P0A6Y8	3328.6897	4.51
0.9936	K.RFPLHEM[147.04]RDDVAFQIINDELYLDGNAR.Q	sp|P69908	3262.5827	4.52
1	K.EAYELVAPILTK.I	sp|P00350	1345.7497	4.53
0.9999	K.GNTGENLLALLEGR.L	sp|P0A7V8	1455.7677	4.53
0.9996	K.STAESIVYSALETLAQR.S	sp|P02359	1837.9417	4.53
0.9999	K.ISYISTGGGAFLEFVEGK.V	sp|P0A799	1873.9457	4.53
1	K.TQLPSGSELSLYDIAPVTPGVAVDLSHIPTAVK.I	sp|P61889	3374.7977	4.53
1	K.STAESIVYSALETLAQR.S	sp|P02359	1837.9417	4.53
1	R.GIEEVGPNNVPYIVATITSNSAGGQPVSLANLK.A	sp|P0A853	3308.7257	4.53
1	R.GIEEVGPNNVPYIVATITSNSAGGQPVSLANLK.A	sp|P0A853	3308.7257	4.53
1	K.AALESTLAAITESLK.E	sp|P0ACF0	1516.8347	4.53

1	K.FESEVYILSK.D	sp|P0CE47	1213.6227	4.53
0.9994	R.EM[147.04]LPVLEAVAK.A	sp|P0A6F5	1214.6577	4.53
0.9997	K.AALELAEQR.E	sp|P0A7V8	999.5347	4.53
1	R.LTGLEGEQLGIVSLR.E	sp|P0A707	1583.8887	4.53
0.9716	K.IEIEAIAVR.R	sp|P0AF93	1012.5917	4.53
0.992	K.TVPM[147.04]FNEALAELNK.I	sp|P0A7V0	1591.7917	4.53
1	K.AM[147.04]VEVFLER.G	sp|P0A825	1108.5587	4.53
0.9993	K.QAIVAEVSEVAK.G	sp|P0A7J3|	1242.6817	4.53
0.9995	K.NIEFFEAR.R	sp|P0A7R1	1024.4977	4.53
1	R.VPEGIGETAIVQIR.N	sp|P27248	1480.8247	4.53
0.3364	R.AAVEEGVVAGGGVALIR.V	sp|P0A6F5	1566.8727	4.53
1	R.VATEFSETAPATLK.S	sp|P0A799	1463.7507	4.53
0.9999	R.LATLPTYEEAIAR.L	sp|P0A7J3|	1446.7717	4.53
0.7658	K.LEVVVNER.R	sp|P0ACF8	956.5287	4.53
0.9984	K.GLNVMQNLLTAHPDVQAVFAQNDEM[147.04]ALGAL	sp|P02925	3351.6707	4.54
1	R.HASDDEPFSALAFK.I	sp|P0A6M8	1533.7097	4.54
1	K.ITFIDGDEGILLHR.G	sp|P0ABH7	1597.8467	4.54
0.9999	K.VFVADEYTM[147.04]VYSHIDR.I	sp|Q46938	1959.9037	4.54
0.3464	R.E[111.03]AATHAADAADSAR.A	sp|P76072	1337.5957	4.54
1	K.GLNVM[147.04]QNLLTAHPDVQAVFAQNDEM[147.0	sp|P02925	3367.6657	4.54
0.9525	K.AKIELSSAQQTDVNLPYITADATGPK.H	sp|P0A6Y8	2730.4077	4.56
0.9945	R.AIDKPFLLPIEDVFSISGR.G	sp|P0CE47	2116.1567	4.56
0.9925	R.ILTGDKVTVELTPYDLSK.G	sp|P69222	1991.0827	4.56
0.9999	R.AIDKPFLLPIEDVFSISGR.G	sp|P0CE47	2116.1567	4.56
0.9988	K.LTKPVELIATLDDSAK.S	sp|P35340	1712.9557	4.56
0.9178	K.QDVPSFRPGDTVEVK.V	sp|P0A7K6	1672.8417	4.56
0.9997	K.LQTLGLTQGTVVTISAEGEDEQKAVEHLVK.L	sp|P0AA04	3192.6877	4.57
0.9936	K.LGVLGFEVDHER.N	sp|P0A6A3	1369.6987	4.65
0.998	R.ELGVINIGGAGTITVDGQC[160.03]YEIGHR.D	sp|Q46938	2628.2967	4.65
0.8833	K.DLEHPIEVPVGK.A	sp|P0ABB4	1331.7087	4.65
1	R.SGKSELEAFEVALENVRPTVEVK.S	sp|P02359	2530.3277	4.65
0.9754	R.LGANPVPLQLAIGAEEHFTGVVDLVK.M	sp|P0A6M8	2686.4697	4.65
1	R.ILENGEVKPLDVK.V	sp|P0A6F9	1452.8187	4.68
0.9962	K.TLDTQGLRNEFLVEK.V	sp|Q46938	1761.9257	4.68
1	K.TQLIDVIAEKAELSK.T	sp|P0ACF0	1656.9297	4.68
0.9987	K.DGISYTFSIVPNALGKDDEVRK.T	sp|P09373	2423.2337	4.68
0.9963	K.STLTPVVISNM[147.04]DEIKELIK.L	sp|P69783	2145.1607	4.68
0.9956	K.LGPYEFIC[160.03]TGRPDEGIPAVC[160.03]FK.L	sp|P69908	2525.2087	4.68
0.999	K.WKEGEATLAPSLDLVGKI.-	sp|P0AE08	1926.0467	4.68
0.9795	R.AKLESLVEDLVNR.S	sp|P0A6Y8	1484.8197	4.68
0.9978	R.NHETGELLATFELK.T	sp|P36683	1600.8097	4.75
0.9998	R.GNPTVEAEVHLEGGFVGM[147.04]AAAPSGASTGSR.E	sp|P0A6P9	2871.3457	4.75
1	R.ELPELTAEFIKR.F	sp|P0A850	1444.7927	4.79
0.675	K.E[111.03]VLIFGGQEVETKR.F	sp|P60752	1585.8467	4.79
1	R.EVPVEVKPEVR.V	sp|P68066	1279.7137	4.79
1	K.HEDM[147.04]YTAINELINKLER.Q	sp|P0AD49	2104.0257	4.83
0.9999	R.GYLSPYFINKPETGAVELESPFILLADKK.I	sp|P0A6F5	3238.7167	4.87
0.619	K.Q[111.03]LPC[160.03]PAELLR.L	sp|P0A853	1178.6117	5.99
0.5839	E.LSKTQAKAALESTLAAITESLK.E	sp|P0ACF0	2273.2847	8.5
0.4233	R.GQSSAALALGM[147.04]THWQSM[147.04]K.L	sp|P0AER5	1934.8977	8.76
0.5485	R.VHLPPLRERR.E	sp|P0AFB8	1271.7577	11.7

LC-MS/MS result obtained from the fraction No.2 (middle well, where the sample was loaded) after OFFGEL fractionation of an *E.coli* protein digest.

probability	peptide	protein	calc_neutral_pep_mass	pI
0.1577	K.Q[111.03]SSAALESYLN.R	sp|P37325|YBCH_ECOI	1164.5297	4
0.1626	K.IGTGATQA.S	sp|P31678|OTSB_ECOI	717.3657	5.52
0.1659	K.NANETKSGAG.Q	sp|P08957|T1MK_ECO	947.4307	6
0.1717	K.VTKIFV.D	sp|P61175|RL22_ECOL	705.4427	8.72
0.1965	K.ILAFAHI.M	sp|P0AAD6|SDAC_ECO	783.4647	6.74
0.207	K.VTKIFV.D	sp|P61175|RL22_ECOL	705.4427	8.72
0.2104	K.GDIGAFD.S	sp|P76104|YDCP_ECOI	693.2967	3.89
0.2105	R.VSSPM[147.04]VA.A	sp|P30176|YBIA_ECOL	705.3367	5.49
0.3067	K.NIIPQFRIP.A	sp|Q46811|YGFK_ECO	1096.6397	9.75
0.3439	K.Q[111.03]NLPGAEEGD.G	sp|P36929|RSMB_ECO	1011.4147	3.88
0.3489	Q.Q[111.03]DDM[147.04]TK.N	sp|P0A705|IF2_ECOLI,:	735.2747	4.21
0.3823	A.GPFGKSDR.A	sp|P76090|YNBA_ECO	862.4297	8.75
0.5704	K.FGM[147.04]AGGS.I	sp|P37645|YHJG_ECOL	641.2477	5.52
0.5828	K.GMIATGEG.T	sp|P10906|UGPE_ECO	734.3267	4
0.7303	-.MKVGFIGLGIM[147.04].G	sp|P0ABQ2|GARR_ECC	1180.6347	8.5
0.7998	K.LLTWGFRFFE.T	sp|P0AEB2|DACA_ECO	1314.6757	6.22
0.8364	R.NLTNRHIQLIA.I	sp|P0AAE0|CYCA_ECO	1291.7357	9.76
0.8387	R.GVRQLLELDP.G	sp|P31802|NARP_ECO	1138.6347	4.37
0.8653	R.HSGMESR.Q	sp|P60869|YBJL_ECOLI	802.3387	6.75
0.8842	R.Q[111.03]GKASVND.A	sp|P38035|RTCR_ECOI	800.3667	6.09
0.9809	R.LPNNFWVPR.C	sp|P0A8P1|LFTR_ECOL	1141.6037	9.75

LC-MS/MS result obtained from the fraction No.3 (cathodic side) after OFFGEL fractionation of an *E.coli* protein digest

probability	peptide	protein	calc_neutral_pep_mass	pI
0.7346	K.NMITGAAQMDGAILVVAATD	sp|P0CE47|EFTU1_ECOI	2728.3347	4.21
0.9976	R.TLTLSGMLAEAIR.R	sp|P0A717|KPRS_ECOLI	1374.7537	5.66
0.891	Y.TAINELINKLER.Q	sp|P0AD49|RAIA_ECOLI	1412.7987	5.81
0.9924	W.TLLTELLKEIPAK.-	sp|P15288|PEPD_ECOLI	1467.8917	5.81
0.9981	Y.VVPAFTGLGAPYWDPYAR.G	sp|P0A6F3|GLPK_ECOLI	1978.9937	5.81
0.9998	K.IQGIGAGFIPANLDLK.L	sp|P0ABK5|CYSK_ECOLI	1625.9137	5.84
0.9999	K.IATDPFVGNLTFFR.V	sp|P0A6M8|EFG_ECOLI	1596.8297	5.84
0.9004	R.GVNVLADAVK.V	sp|P0A6F5|CH60_ECOLI	984.5607	5.84
0.9953	K.GGTRIPISGIAGDQQAALF.G	sp|P0A6F3|GLPK_ECOLI	1870.9897	5.84
0.9882	K.AADIVLQAAIAAGAPK.D	sp|P0A9Q7|ADHE_ECOI	1478.8457	5.88
0.9986	K.AGIALNDNFVK.L	sp|P0A9B2|G3P1_ECOLI	1160.6187	5.88
0.9997	K.AGPLAGYPVVDMGIR.L	sp|P0A6M8|EFG_ECOLI	1514.7917	5.88
0.999	K.KYDIPVVMDSAR.F	sp|P0A853|TNAA_ECOL	1392.7067	5.96
1	K.LLDNAAADLAAISGQKPLITK.A	sp|P62399|RL5_ECOLI	2122.1997	5.96
0.9226	L.IMDKIYDVLR.A	sp|P0C8J8|GATZ_ECOLI	1264.6847	5.96
0.9976	R.KIDGIPALLDR.A	sp|P37769|KDUD_ECOL	1209.7077	5.96
0.6715	F.LVDLVDKNLTGK.E	sp|P0A825|GLYA_ECOLI	1313.7557	5.96
0.8699	Y.VVVAALGELAK.R	sp|P0AFG8|ODP1_ECOL	1068.6547	5.97
0.9999	K.VILAGEVTTPVTVR.G	sp|P02413|RL15_ECOLI	1453.8507	5.97
0.9697	K.VLPELNGKLTGMAF.R	sp|P0A9B2|G3P1_ECOLI	1488.8007	5.97
1	R.VVVGLLLGEVIR.T	sp|P0A850|TIG_ECOLI	1265.8067	5.97
0.995	K.VIVEGINLVK.K	sp|P60624|RL24_ECOLI	1082.6697	5.97
1	R.VFMQPASEGTGIIAGGAMR.A	sp|P0A7W1|RS5_ECOLI	1891.9287	5.97
0.9999	R.GSVNPEC[160.03]TLAQLGAA	sp|P0A9G6|ACEA_ECOL	1614.8037	5.99
0.9934	K.QLPC[160.03]PAELLR.L	sp|P0A853|TNAA_ECOL	1195.6387	5.99
0.9997	R.FGGESVLAGSIIVR.Q	sp|P0A7L8|RL27_ECOLI	1403.7777	6
0.9998	R.GAEQIYIPVLIK.K	sp|P0A853|TNAA_ECOL	1342.7857	6
0.9998	R.GAEQIYIPVLIK.K	sp|P0A853|TNAA_ECOL	1342.7857	6
0.9998	R.LQAFEGVVIAIR.N	sp|P0A7K6|RL19_ECOLI	1314.7657	6
0.986	R.FAENAYFIK.Q	sp|P0A853|TNAA_ECOL	1101.5497	6
0.9991	K.FNQIGSLTETLAAIK.M	sp|P0A6P9|ENO_ECOLI	1604.8777	6
0.9993	K.GGFTVELNGIR.A	sp|P0AG67|RS1_ECOLI	1161.6137	6
0.9963	R.GAEQIYIPVLIK.K	sp|P0A853|TNAA_ECOL	1342.7857	6
0.9898	Y.AAIDGLIDKLAR.Q	sp|P0AFX0|HPF_ECOLI	1254.7297	6
0.8134	R.LQAFEGVVIAIR.N	sp|P0A7K6|RL19_ECOLI	1314.7657	6
0.9928	K.GLTFTYEPK.V	sp|P0A853|TNAA_ECOL	1054.5337	6
0.9906	K.LFNELGPR.F	sp|P0AG44|RL17_ECOLI	944.5077	6
0.9666	K.GLTFTYEPK.V	sp|P0A853|TNAA_ECOL	1054.5335	6
0.9991	K.LAEVLAAANAR.A	sp|P0A7R1|RL9_ECOLI	1097.6197	6
0.9999	R.ALLNSMVIGVTEGFTK.K	sp|P0AG55|RL6_ECOLI	1678.8967	6.05
0.9994	K.ALGANLVLTEGAK.G	sp|P0ABK5|CYSK_ECOLI	1255.7137	6.05
0.9945	R.AGENVGVLLR.G	sp|P0CE47|EFTU1_ECOI	1026.5817	6.05
0.9721	R.AGENVGVLLR.G	sp|P0CE47|EFTU1_ECOI	1026.5817	6.05
0.9797	W.ATQSSTLVEVLAK.A	sp|P0A6F3|GLPK_ECOLI	1345.7457	6.05
0.9965	K.AANIIGIQIEFAK.V	sp|P02413|RL15_ECOLI	1386.7867	6.05
0.9932	R.KLEDLLAAKL.-	sp|P0A867|TALA_ECOLI	1112.6807	6.07
0.9987	K.ITLNMGVGEAIADKK.L	sp|P62399|RL5_ECOLI	1558.8387	6.07
0.8173	Y.KFNGWELDINSR.S	sp|P0A9Q1|ARCA_ECOL	1477.7317	6.07
0.8123	R.KAVEFQDILK.M	sp|P0AC38|ASPA_ECOLI	1189.6707	6.07
0.9995	R.LRGWQVPAFTLGGEATDIVVN	sp|P69908|DCEA_ECOLI	2415.2737	6.07
1	R.KVVADIAGVPAQINIAEVR.K	sp|P0A7V3|RS3_ECOLI	1962.1257	6.07

0.9998	R.LKGNTGENLLALLEGR.L	sp|P0A7V8|RS4_ECOLI	1696.9467	6.14
0.9799	R.KISWMEIYTGEK.S	sp|P08200|IDH_ECOLI	1483.7377	6.14
0.944	F.THAGTENLLALVSAM[14	sp|P0C8J6|GATY_ECOLI	1741.9037	6.41
0.9979	F.THAGTENLLALVSAMAK.I	sp|P0C8J6|GATY_ECOLI	1725.9087	6.41
0.9865	R.MVQLFFPDPWHK.A	sp|P0A8I5|TRMB_ECOLI	1543.7647	6.5
0.8306	-.MENFKHLPEPFR.I	sp|P0A853|TNAA_ECOLI	1543.7607	6.51
0.9995	K.VLDLIAHISK.-	sp|P0A9B2|G3P1_ECOLI	1107.6647	6.71
0.8149	A.VEPHAVATLSC[160.03]I	sp|P39361|SGCR_ECOLI	1423.7497	6.71
0.999	R.VFQTHSPVVDSISVK.R	sp|P0A7K6|RL19_ECOLI	1641.8727	6.71
0.7811	K.WMYHYC[160.03].C	sp|P36979|RLMN_ECOLI	958.3467	6.73
0.8847	R.DGHPLFAGFVK.A	sp|P0A7E5|PYRG_ECOLI	1186.6137	6.74
0.9113	R.GGVSLRPGDGVIHSW.L	sp|P36683|ACON2_ECOL	1535.7847	6.74
0.9437	R.HVAILGDLQGPK.I	sp|P21599|KPYK2_ECOLI	1246.7037	6.74
0.9983	K.GPFIDLHLLK.K	sp|P0A7U3|RS19_ECOLI	1151.6707	6.74
0.8063	R.RVAALEGDVLGSYQHGA	sp|P0A6P1|EFTS_ECOLI	1897.9757	6.75
0.6622	K.AIQLHPLVC[160.03]AAY	sp|P0A8T7|RPOC_ECOLI	1354.7067	6.77
0.9394	R.ALPLPVSVPSHC[160.03]	sp|P0AAI9|FABD_ECOLI	1459.7857	6.78
0.9999	R.AVLEVAGVHNVLAK.A	sp|P0A7W1|RS5_ECOLI	1418.8247	6.79
0.9997	R.AVLEVAGVHNVLAK.A	sp|P0A7W1|RS5_ECOLI	1418.8247	6.79
0.9899	R.KVLNIFPSIDTGVC[160.0	sp|P0A862|TPX_ECOLI	2046.0927	8.22
0.9917	R.SNTGLVIDPYFSGTKVK.V	sp|P0A6F3|GLPK_ECOLI	1824.9617	8.22
0.9983	K.TGKYDAVIALGTVIR.G	sp|P61714|RISB_ECOLI	1575.8987	8.25
0.9923	Y.SNKVLDLIAHISK.-	sp|P0A9B2|G3P1_ECOLI	1436.8347	8.33
0.9998	K.TTLTAAITTVLAK.T	sp|P0CE47|EFTU1_ECOLI	1302.7757	8.41
0.9737	K.TPPAAVLLK.K	sp|P0A7J7|RL11_ECOLI	908.5697	8.41
0.7166	L.TAAITTVLAK.T	sp|P0CE47|EFTU1_ECOLI	987.5967	8.41
0.9997	K.SAGGIVLTGSAAAK.S	sp|P0A6F9|CH10_ECOLI	1201.6667	8.47
0.9675	Y.SHIDRIIVGGIMPITK.T	sp|Q46938|KDUI_ECOLI	1748.9967	8.49
0.8914	-.MITGIQITK.A	sp|P68066|GRCA_ECOLI	1003.5737	8.5
0.9996	R.GAEQIYIPVLIKK.R	sp|P0A853|TNAA_ECOLI	1470.8807	8.5
0.945	-.MKVAVLGAAGGIGQAL.A	sp|P61889|MDH_ECOLI	1454.8277	8.5
0.8538	K.KLQLVGVGY.R	sp|P0AG55|RL6_ECOLI	975.5757	8.59
0.9899	K.KLADSGLNIIAAK.G	sp|P0A836|SUCC_ECOLI	1312.7717	8.59
0.914	K.GKPFAPLLEK.N	sp|P60422|RL2_ECOLI	1098.6437	8.59
0.9974	K.FVNILMVDGKK.S	sp|P02359|RS7_ECOLI	1262.7057	8.59
0.9998	R.GVNVLADAVKVTLGPK.G	sp|P0A6F5|CH60_ECOLI	1579.9297	8.59
0.9989	K.KGPFIDLHLLK.K	sp|P0A7U3|RS19_ECOLI	1279.7657	8.6
0.6623	R.AANKFPAIIY.G	sp|P68919|RL25_ECOLI	1106.6127	8.63
0.7071	K.VKAPVIVQF.S	sp|P0AB71|ALF_ECOLI	999.6117	8.72
0.9671	W.VGQGATISDRVAALIK.E	sp|P0A7T3|RS16_ECOLI	1597.9147	8.72
0.9996	K.IAAANVPAFVSGK.A	sp|P0ACF0|DBHA_ECOLI	1243.6927	8.75
0.9743	Y.GVAIADGPLKGLAAR.A	sp|P0A862|TPX_ECOLI	1407.8197	8.75
0.9907	K.KLNSAVFPGGQGGPL.M	sp|P0A825|GLYA_ECOLI	1440.7727	8.75
0.9916	Y.IVATITSNSAGGQPVSLAI	sp|P0A853|TNAA_ECOLI	2040.1217	8.75
0.9987	Y.IVATITSNSAGGQPVSLAI	sp|P0A853|TNAA_ECOLI	2040.1217	8.75
0.9997	K.IAAANVPAFVSGK.A	sp|P0ACF0|DBHA_ECOLI	1243.6927	8.75
0.9997	R.IIVGGIMPITK.T	sp|Q46938|KDUI_ECOLI	1140.6937	8.75
0.9092	R.IILLGAPGAGKGTQAQF.I	sp|P69441|KAD_ECOLI	1640.9247	8.75
0.9359	R.DVILFPAMRPVK.-	sp|P0A8N3|SYK1_ECOLI	1384.7897	8.75
0.9992	K.AYGSTNPINVVR.A	sp|P0A7W1|RS5_ECOLI	1289.6728	8.79
0.6968	K.EITIAAAKVPSFR.A	sp|P0ACF4|DBHB_ECOLI	1401.7977	8.85
0.9064	R.SQWLGGWR.E	sp|P0AFW2|RMF_ECOLI	988.4877	9.47

0.9987	R.AFLPGSLVDVRPVR.D	sp|P0AG67|RS1_EC	1524.8777	9.64
0.9345	Q.KKAEAAAAALK.K	sp|P19934|TOLA_E	1070.6447	9.7
0.9347	Q.KKAEAAAAALK.K	sp|P19934|TOLA_E	1070.6447	9.7
0.9899	K.VQLLGSGSILR.H	sp|P0AFG8|ODP1_	1141.6817	9.72
0.9752	R.VVGQLGQVLGPR.G	sp|P0A7L0|RL1_EC	1221.7197	9.72
0.9996	R.VVGQLGQVLGPR.G	sp|P0A7L0|RL1_EC	1221.7197	9.72
0.8168	R.RAVQLNSLSGF.C	sp|P0A7D4|PURA_	1190.6407	9.75
0.9684	K.ITVVPILR.A	sp|P0A8F0|UPP_EC	909.6007	9.75
0.687	Y.KPGNVVLTPTILR.D	sp|P0AB71|ALF_EC	1406.8607	11
0.7176	T.PVTRGVR.V	sp|P75779|YBIX_EC	783.4717	12

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Chapter IV.

Electro-elution device for protein/peptide purification using ultra narrow pH gradient gels

1. Introduction

One of the main goals of proteomics is to identify and characterize the structures, functions, biological activities, post-translational modifications and interactions of proteins occurring in a given cellular organism. The main challenge in proteomics is to deal with complex matrix samples that can prevent easily the presence of low abundant target proteins. Therefore, sample pretreatment and protein purification are often required to make possible the extraction of reliable information about different protein features such as biological activity, size, binding affinity, molecular weight, charge, *etc*.[1] Information about different physicochemical properties of the proteins can be achieved by using high resolving power chromatographic techniques such as gel-filtration chromatography based on the separation of molecules by size[2], ion-exchange chromatography where the proteins are separated according to their charge[3], hydrophobic interaction chromatography[4], and affinity chromatography based on the trapping of the target proteins by using their affinity to specific chemical groups[5]. However, the most widely used and well-known technique for protein fractionation is two-dimensional (2D) gel electrophoresis. In isoelectric focusing (IEF), the proteins are separated by their isoelectric points using an immobilized pH gradient (IPG) gel. Most often IEF is used as the first dimension of two-dimensional (2D) gel electrophoresis where the proteins are then separated by molecular mass using sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE)[6]. 2D gel electrophoresis is commonly followed by in-gel proteolysis of a selected band of proteins and a subsequent mass spectrometry (MS) analysis[7-9]. In general, chemical analysis of proteins using electrophoretic methods coupled with MS detection involve three important steps: separation of the protein mixture, staining the gel slab[10, 11] or protein transfer from the gel to a different liquid or solid support and finally, MS detection. Protein extraction from the gel can be achieved by several strategies. Extraction by diffusion or migration (e.g. passive elution, blotting or electro blotting) is the simplest way to extract molecules from the gel, achieved by adding an aqueous solution (buffer) to the gel and forcing the proteins (*e.g.* by pressure or an electric field) to move out from the gel. Alternatively extraction may be achieved by applying a voltage across the gel, so called active elution [12]. Protein extraction can be directly implemented into electro-elution devices; several of them have been reported in literature[13-16]. Among the main factors to consider during the purification step is to

restrict the loss of sample, achieve reproducibility, limit the time of extraction and maximize the recovery of the proteins.

One of the solutions to increase the separation efficiency, to decrease the time of analysis, prevent sample consumption and minimize the cost of analysis *etc.* may be considered by the use of microfluidic systems. Microscale liquid flow and sample consumption may be controlled by efficient pumping systems using microfluidic-based devices[17]. Coupling the microfluidic system with isoelectric focusing and direct elution of proteins/peptides from the gel slab provides a promising strategy to avoid time-consuming steps of gel sectioning[18] and could be used for clinical applications[19].

Despite recent improvements in instrumentation and technology, these techniques listed above are still far from fully characterizing the proteome in the cell, meaning that there is a big interest on developing new scientific tools and concepts allowing to simplify, purify, identify, characterize and quantify the proteins[20].

It should be mentioned that the work presented in this chapter has been done at the end of my PhD and that the results should be considered as a preliminary proof-of-concept and that there is ample room for improving the design of the device. Some improvement avenues are proposed in Chapter IX.

In this part, an electro-elution device enabling the direct extraction of protein/peptide samples from an immobilized ultra narrow pH gradient (UNPG) gel is proposed. The molecules extracted from the gel by the elution buffer could be analysed by MS (MALDI or ESI) or may be applied for further investigations. UV-vis spectroscopy was performed for protein quantification. The electro-elution device was employed for rapid and effective separation of the analytes based on their isoelectric points and afterwards coupled to MS. The proposed approach is low cost, fast, easy to use and can be reproduced in any laboratory.

2. Material and Methods

2.1 Chemicals & Materials

Immobilized pH gradient gel (Immobiline™ Dryplate pH 4 to 7) was purchased from Amersham Biosciences (Uppsala, Sweden). Cytochrome c from horse heart (12.3 kDa), myoglobin from horse skeletal muscle (17.0 kDa), lysozyme (14.3 kDa), bovine serum albumin (BSA, 66.4 kDa) were obtained from Sigma-Aldrich (Schnelldorf, Switzerland). Trypsin (bovine pancreas, 23.8 kDa) was from AppliChem GmbH (Darmstadt, Germany). Iodoacetamide (IAA) and 1,4-dithiothreitol (≥ 99%) were purchased from Fluka. Deionised water was purified by an alpha Q Millipore system (Zug, Switzerland) and used in all aqueous solutions. The UV-visible absorption spectra were obtained with a standard spectrophotometer (Perkin Elmer, model Lambda XLS+) using quartz cells with a path length of 1 cm. A calibration BCA protein assay kit for determining protein concentrations was obtained from Thermo Scientific (Rockford, USA).

2.2 BSA digestion

40 mL of 100 mM ammonium bicarbonate (NH_4HCO_3) was added to 100 mL of bovine serum albumin solution (87 mM). Afterwards, 5 mL of 200 mM 1,4-dithiothreitol (DTT) was added and the mixture was incubated at 95 °C for 5 min. 10 mL 200 mM iodoacetamide (IAA) was tacked and the solution was incubated for 20 min at room temperature in dark. Finally, trypsin was added with a final ratio of 1:30 (w/w, trypsin: protein), incubated at 37 °C for 20 h. The tryptic digests of BSA was stored under −20 °C.

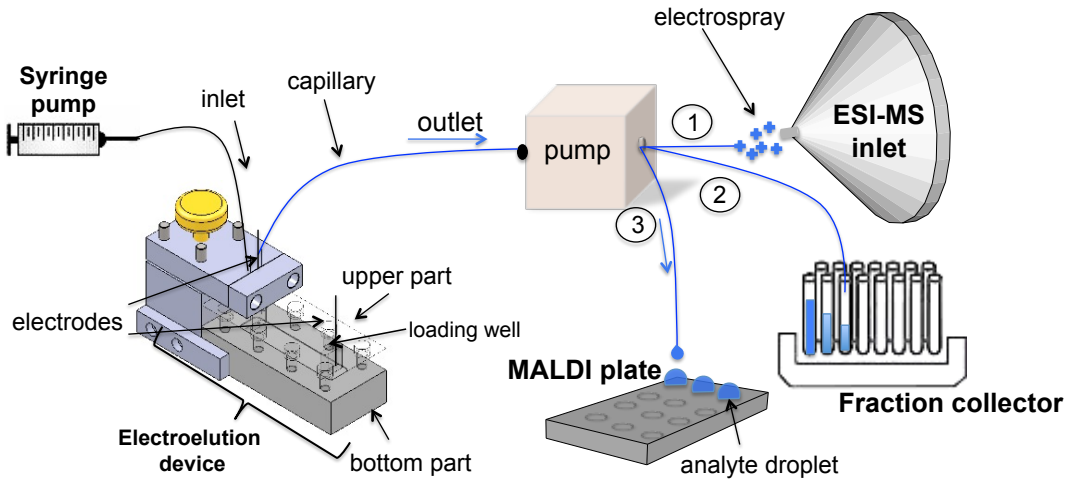
2.3 Protein/peptide purification by electro-elution device

Gel strips (5 cm length, 1 cm width) were cut from Immobiline™ Dryplate pH 4 to 7 and re-swollen in water for one hour before their use. Depending on the sample charge, the gel strips were cut from the regions of the gel of specific pH, in such a way that an ultra narrow pH gradient gel is obtained. The electro-elution device consists of two compartments: upper and bottom (see Scheme 4.1). At the beginning of each experiment an UNPG gel strip of appropriate length is placed into the bottom tray of electro-elution device, upper part is placed on top and fixed. From both sides

of the device there are two compartments; one is for sample loading, another for its extraction. Next to these open cells two Pt electrodes are placed thus to be in contact with the gel. Two silica capillaries (with inner diameter ID: 150 μm) were inserted into the second open channel (1 cm length, 0.7 cm height) to load the elution buffer using the syringe pump and to collect the extracted sample by peristaltic pump. When the sample is loaded on top of the gel and the electric field is applied, the sample starts to migrate to the electrode whereas the syringe pump (KD Scientific, 250 μl Gastight syringe 1700 series, Hamilton) starts pushing the aqueous buffer to the channel through the silica capillary and the peristaltic pump (IPC-N 24, Ismatec, equipped with Tygon tubing) starts pulling the liquid from the same well. The solutions pumped out from the extraction well are collected into the vials every regular time intervals and further analysed by mass spectrometry or any other analytical technique.

Fractionation of cytochrome c, lysozyme and myoglobin was performed using a gel with a narrow pH gradient in a range of 4.00-4.27 under the following voltage program (150 V for 40 min, 700 V for 3h) and limited current of 1 mA with an EPS 3501 XL power supply (Amersham Pharmacia Biotech, Sweden).

The BSA digest was separated and purified using the gel with a pH range of 4.00-4.27 under following conditions: 150 V for 30 min, 700 V for 3 and 1000 V for 30 min with a maximum current of 2 mA.



Scheme 4.1. Schematic representation of the experimental setup for electro-elution of proteins from the gel media. The liquid is pumped *via* fused silica capillaries by a syringe pump to an external pump (liquid flow directions are indicated with arrows). Sample detection is performed using either ESI-Ms or MALDI-MS tools.

2.4 MS analysis

The protein analysis was performed using a Microflex MALDI-TOF instrument (Bruker Daltonics) equipped with a 337 nm nitrogen laser. 2 μL of the extracted protein solution was deposited on a steel target plate with 2 μL of sinapinic acid (SA) matrix (15 mg/mL Sinapinic acid in 50% acetonitrile, 0.1% Trifluoroacetic acid and 49.9% water) and left to dry at room temperature. In case of peptide analysis DHB matrix (10 mg/mL 2,5-dihydroxybenzoic acid in in 50% acetonitrile, 0.1% Trifluoroacetic acid and 49.9% water) was used. For the analysis of BSA digest, the mass-to-charge ratios of peaks were compared with the molecular weights of all the possible peptides generated from BSA by trypsin digestion. On-line tools of FindPept and Compute pI/Mw from ExPASy (www.expasy.org) were used to help the comparison and p*I* identification.

On-line coupling of the electro-elution device with electrospray ionization mass spectrometry (ESI-MS) was performed on the Thermo LTQ Velos. For MS experiments, 3.7 kV of voltage was applied to induce electrospray ionization and to generate *m*/*z* spectrum.

3. Results and discussion

The electro-elution device was employed for rapid protein separation based on their isoelectric points using immobilized UNPG polyacrylamide gels. The principle of the electrophoretic fractionation in an ultra narrow pH gradient gel media is described in Chapter III[21]. In brief, the positively charged proteins or peptides with p*I*'s higher than the pH of the gel migrate to cathode, while the negatively charged molecules with p*I*'s lower than the gel pH migrate to the anode. Differing on the sample charge, the electrode polarity might be changed depending on molecules of interest that should be extracted. In the presence of an electric field, the charged species start to migrate to the opposite electrode at a different speed. The theoretical distance between them can be calculated taking into account the migration velocity (V_m) using equation 4.1:

$$V_{\mathrm{m}} = -\frac{zF}{RT} D\nabla\phi = -zF\tilde{u}\nabla\phi \qquad (4.1)$$

where z is the charge of a protein at a given pH, F is the Faraday constant, R is the gas constant, T is the temperature, D is the diffusion coefficient of the protein and ϕ is the applied electric field.

According to equation 4.1, V_m is a function of the net charge of the protein at a given pH, its diffusion coefficient, the applied electric field and the temperature. Therefore, tuning all these parameters might optimize the separation between two or more proteins. For instance, if two proteins *e.g.* cytochrome c and lysozyme have a similar p*I* and are separated in the same experimental conditions (*i.e.* diffusion coefficient, applied electric field and temperature), the optimum pH of the gel for achieving a defined separation distance within a fixed gel length and in a given experimental time can be calculated. By knowing the time of the separation and the expected separation distance between two proteins, the migration velocity of the proteins could be estimated.

The theoretical calculations fit qualitatively the experimental results (*vide infra*), but not quantitatively, most likely due to inaccuracies on the diffusion coefficient values, since these values reported in the literature vary from the studied media (*e.g.* water or gel) and the specific properties of the media. For instance, it has been shown that the diffusion coefficient of proteins is higher when diffusing inside gels with carrier ampholytes than with fixed immobilines as a consequence of the salt formed between the proteins and the polymeric coils that contains the immobilines. According to Righetti *et al.* differences up to three-folds can be found between diffusion coefficients of the same protein measured in polyacrylamide gels with the same porosity but with different concentrations of immobilines close to their p*I* zone[22]. It is expected that interactions between proteins and its surroundings are more evident far away from the p*I* zone of the protein. As a consequence, it is not surprising to find differences between the calculated and the experimental results (calculated diffusion coefficient 35 times smaller than the one reported in the literature[23]). However, by taking into account the experimental results (*i.e.* the position of the migrated proteins), the diffusion coefficient of the different proteins can be predictable in the present conditions and employed for future predictions.

Table 4.1. Parameters employed for the calculation of the migration velocity and the separation distance between cytochrome c (p*I*=9.6) and lysozyme (p*I*=9.1).

	Cyt c, 12300 Da	Lys, 14300 Da
V /*l* m^{-1}	14000	14000
Z^+(pH=4)	20.7	16.5
Diffusion Coef. /m^2 s^{-1}	2.25E-12*/ 8.00E-11**	2.40E-12*/ 8.20E-11**
T /K	298	298
Migration Vel /cm s^{-1}	2.54E-03	2.16E-03
Time /s	360	496.8
ΔT/s	136.8	
Migrated distance /cm	0.91	0.78
Separation dist. /cm	0.14	

* Obtained values from the experimental results

** Reported in literature

It was estimated that the fastest protein migration would be achieved at pH values close or around 4, the separation distance between two proteins is 1.4 mm.

When the protein with the highest p*I* value would reach the elution well, the molecules would be extracted from the gel by the elution buffer and drawn out using the silica capillary (ID: 150 μm) connected to peristaltic pump (Scheme 4.1). The optimal flow rates used during the experiments for syringe and peristaltic pump were calculated using the following equation (eq. 4.2):

$$VF = \pi * \left(\frac{Cid}{2}\right)^2 * \frac{LF}{60} \quad (4.2)$$

where C_{id} is the inner diameter of the capillary in cm, *LF* correspond to linear flow rate (cm/h).

Drawn out samples of proteins/peptides were further employed for quantification using UV-Vis spectroscopy and for MS analysis.

3.1 Elution of protein mixture for MALDI-MS analysis

The mixture of three positively charged (at pH= 4.0) proteins cytochrome c (p*I*=9.6), lysozyme (p*I*=9.1) and myoglobin (p*I*= 7.0) 0.26 μg/mL each was loaded on top of the ultra narrow gel having the pH 4.00-4.27 through the filling well and the electric potential of 150V for 40 min, 700V for 3h with limited current of 1 mA was applied.

The speed at which the proteins migrate is determined by their size and mass, meaning that cytochrome c would be detected first (z^+=20.1 pH=4.1 and M.W= 12300) and myoglobin the last due to the higher charge at the selected pH gradient (z^+=25.0 pH=4.1 and M.W= 17000).

The flow rates used for 20 mM TRIS-HCL buffer delivering to the extraction well and its pulling by the peristaltic pump were 0.7 μl/min.

During the experiment, the fractions containing 20 mM TRIS-HCl buffer (pH=7.0) with or without protein sample were collected every 2 min for 3 h to an Eppendorf tube, and then deposited on the steel plate for further MALDI-MS analysis (Figure 4.1).

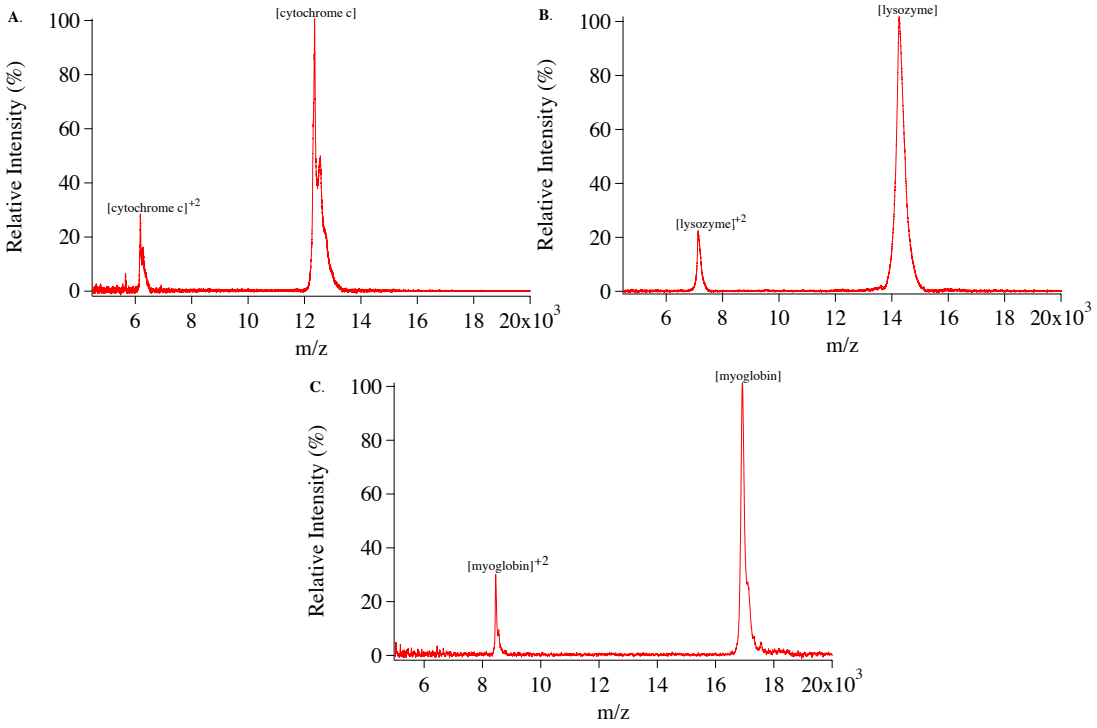


Figure 4.1. Mass spectra of purified proteins collected after A) 6 min, B) 8 min and C) 24 min; ultra narrow gel fractionation of a protein mixture containing cytochrome c, lysozyme and myoglobin. The samples were analyzed by MALDI-MS with sinapinic acid matrix.

Figure 4.1 demonstrates the MALDI-MS spectra of purified protein mixture extracted from ultra narrow pH gel. As it could be seen from MS spectra, all three proteins were extracted separately indicating an important advantage of the electro-elution approach, which is the possibility of purification and localizing of eluted spots directly on the plate for MS analysis. Cytochrome c was detected in 6 min, lysozyme in 8 min and myoglobin in 24 min; applying higher potential can reduce the time of analysis, however this may lead to the gel burning due to Joule heating.

For the quantification of proteins recovery UV-Vis spectroscopy was employed. All experiments were repeated three times and the results are summarized in Table 4.2.

Table 4.2. Protein recovery values determined after electrophoretic separation in electro-elution device over an immobilized ultra narrow pH gel (pH 4-4.27) using UV-spectroscopy.

Sample	Cytochrome c (p*I* = 9.6)	Lysozyme (p*I* = 9.1)	Myoglobin (p*I* = 7.0)
Protein recovery/%	91,02 ± 1.16%	85,78 ± 3.67%	89, 16 ± 1.96%

According to the results presented in Figure 4.1 and Table 4.1, the proteins were completely fractionated according to their charge/p*I* and recovered with a higher average efficiency of 89 %, that competes with the best electro-elution recovery efficiencies reported in the literature[18, 24]. The fractionation of proteins by using the electro-elution device could be an effective way to filter and isolate proteins from the mixture.

3.2 Elution of protein with online ESI-MS analysis

To demonstrate the feasibility of online coupling of electro-elution setup with ESI MS, the same protein mixture (cytochrome c, lysozyme and myoglobin, 0.026 μg/mL each) was separated using gel with pH gradient (4.00-4.27) placed in the bottom compartment of the device. During the protein migration, the potential program applied was: 150 V for 30 min, then 700 V with a limiting current of 1 mA until the last peak of myoglobin were observed. 10 mM TRIS-HCl buffer was pulled through the silica capillary by the syringe pump with a flow rate of 1.5 μL/min for protein elution. The elution buffer concentration was decreased from 20 to 10 mM in comparison with MALDI experiments, with the aim to diminish interferences due to high salt concentrations. However, the flow rate used for both the syringe and the peristaltic pumps was increased twice in order to obtain a stable and high *m/z* signal from the proteins. The silica capillary connected to the commercial ion source of Thermo LTQ Velos mass spectrometer was pulling out the liquid with a linear speed of 5.7×10^{-2} m/s. Figure 4.2 represents the mass spectra of proteins identified during the protein fractionation followed by online ESI-MS detection. In Figure 4.A and C. the single peaks of cytochrome c and myoglobin are observed, however on Figure 4.2B both cytochrome c and lysozyme were detected, due to the mixing taking place in the capillary.

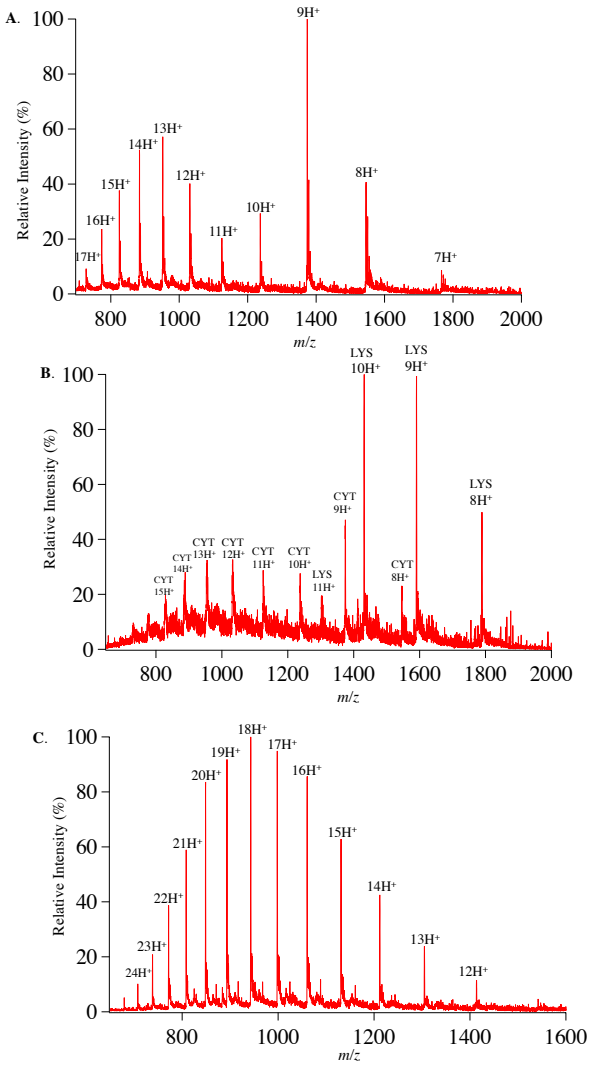


Figure 4.2. Mass spectra of A) cytochrome c, B) cytochrome c/ lysozyme mixture, C) myoglobin detected by coupling electro-elution device to ESI-MS. The spectra were obtained in a positive ion mode.

The separation between cytochrome c and lysozyme was not efficient. The latter might be related to a different migration speed among proteins at the chosen pH. In such a case, depending on the gel area where the proteins are introduced, a different net charge and migration velocity will be developed. Also the separation might be not completed due to capillary blocking by re-swollen gel during the experiment causing a varying flow rate during the experiment. The separation between two molecules in the capillary should be at least 5.7 mm in order to be detected as the single peak by ESI-MS, meaning that in our case the distance was much less.

The proposed separation still needs to be improved in order to resolve these two proteins by online coupling of electro-elution device and ESI-MS, nevertheless the result obtained is promising taking into account the close value of the protein p*I*'s.

3.3 MALDI-MS detection of peptide mixture fractionated by an electro-elution device.

To demonstrate the possibility of using an electro-elution device for peptide mixture purification, 10 μL of bovine serum albumin (BSA) tryptic digest 5 mg/mL was introduced to the sample-loading compartment. Gel strip with ultra narrow pH range of 4.00-4.27 was used for peptide separation. The electrode polarity was set in such case that all the negatively charged proteins with a basic p*I*'s should migrate first to the opposite charge electrode. The identification results are summarized in Table 4.3. Amino acids highlighted in black correspond to the ones observed in the several collected samples.

Table 4.3. Peptides identified from BSA digest by MALDI-MS. PTMs: post-translational modifications; Cys_CAM: carbamidomethylation cysteine; M.W.: monoisotopic molecular weight. The p*I* (isoelectric point) was calculated with the Compute pI /Mw tool on ExPASy (http://web.expasy.org/compute_pi/).

After 10 min:

Sequence	PTMs	M.W.	p*I*
QRLR	-	571.68	12.0
KFWGK	-	664.81	10.0
AWSVVAR	-	787.92	9.79
LSQKFPK	-	847.03	10
RHPEYAVSVLLR	-	1439.68	8.75
KVPQVSTPTLVEVSR	-	1639.91	8.75

After 20 min:

Sequence	PTMs	M.W.	p*I*
AWSVAR		664.81	9.79
LSQKFPK		787.92	10
KQTALVELLK		1142.4	8.59
FKDLGEEHFK		1249.39	5.45
HPEYAVSVLLR		1283.49	6.75
SLHTLFGDELCK	CYS_CAM	1419.82	6.74
RHPEYAVSVLLR		1439.68	8.75
KVPQVSTPTLVEVSR		1639.91	8.75

After 40min:

Sequence	PTMs	M.W.	p*I*
QRLR	-	571.68	12.0
AWSVVAR	-	787.92	9.79
LSQKFPK	-	847.03	10
LCVLHEK	CYS_CAM	841.04	6.74
YLYEIAR		927.07	6.0
CCTESLVNR	CYS_CAM	1024.17	5.99
KQTALVELLK		1142.4	8.59
DTHKSEIAHR		1193.28	6.92
FKDLGEEHFK		1249.39	5.45
HPEYAVSVLLR		1283.49	6.75
HLVDEPQNLIK		1305.50	5.3
SLHTLFGDELCK	CYS_CAM	1362.56	5.3
RHPEYAVSVLLR		1439.68	8.75
LGEYGFQNALIVR		1479.70	6.0
EYEATLEECCAK	CYS_CAM	1388.53	4.09
LKECCDKPLLEK	CYS_CAM	1418.73	6.17
KVPQVSTPTLVEVSR		1639.91	8.75

As it can be seen from Table 4.3, firstly the peptides with the basic p*I*'s from 8.75 to 12 were identified after 10 min of separation, after 20 min the peptides with the range of 5.45 to 8.59 were detected by MALDI-MS. Peptides with the acidic p*I* range were detected after 40 min. These results show that depending on the charge and the pH of the gel media, the peptides with basic isoelectric point would migrate to the anode first. However, the mixing of the sample takes place due to the geometry of the device causing the repeatable results, as observed in Table 4.3.

4. Conclusions

Direct elution of protein/peptide mixtures from ultra narrow pH gradient gel using an electro-elution device was proposed. A protein mixture was separated by electrophoretic mobility in an ultra narrow pH gradient gel and afterwards extracted by the elution buffer. It was shown that the collected fractions could be directly deposited to a MALDI plate and analyzed by MALDI–MS or by online coupling with ESI-MS. Protein quantification was performed by UV-spectroscopy. It was shown that good collection efficiency was achieved; the average protein recovery for protein mixture was almost 90%. The electro-elution device provides a fast and effective approach, helping to avoid time-consuming steps of protein extraction from the polyacrylamide gel. Despite the promising results obtained and discussed in the present work, the sample purification in electro-elution device still needs to be enhanced. Step-by-step improvements would be discussed in Chapter IX.

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Chapter V.

Coupling Isoelectric Focusing Gel Electrophoresis to Mass Spectrometry by Electrostatic Spray Ionisation

Based on E. Tobolkina et al. Analytical Chemistry, 2013, 85 (9), 4745-4752. This work was developed in collaboration with L. Qiao.

1. Introduction

System biology and proteomics require advanced analytical strategies. To analyse a large set of biomolecules, various analytical methods are usually combined, including high resolution separation techniques, sensitive detection tools and high throughput data processing software[1]. With the development of soft ionisation strategies, such as matrix-assisted laser desorption/ionisation (MALDI)[2, 3] and electrospray ionisation (ESI)[4, 5], to produce intact molecular ions, mass spectrometry (MS) has been extensively used for characterising the structure of proteins and peptides[1].

Nowadays, the most widely used and developed proteomics strategy is bottom-up proteomics, where the proteins are digested into peptides followed by liquid chromatography (LC)-MS/MS detection[6]. The advantages of this strategy include high throughput and good sensitivity. However, it is also largely limited by the facts that proteins are normally identified based only on the detection of several peptides and that the sequence coverage in general is quite low. This makes the complete structure characterisation of proteins very difficult, especially the post-translational modifications (PTMs)[7].

Jungblut *et al.* have suggested the concept of "protein species", indicating that modifications on proteins lead to new molecules with different functions[7, 8]. Recently, the Consortium for Top Down Proteomics suggested the term "Proteoform" to designate all the molecular forms in which the protein product of a single gene can be found, including changes due to genetic variations, alternatively spliced RNA transcripts and posttranslational modifications[9]. Protein species proteomics or Proteoform requires complete separation of proteins and structure analysis of single protein species. Therefore, top-down proteomics strategies are needed, involving techniques such as high-resolution sample separation, high-resolution MS and fragmentation of protein ions in the gas phase[10]. Compared with bottom-up proteomics, top-down proteomics is limited by the current techniques in analytical chemistry, and suffers the drawbacks such as low throughput and sensitivity. Nevertheless, novel top-down proteomics strategies have already been proposed. To date, Smith and Paša-Tolić have realised the characterisation of protein isoforms and modifications by combining the top-down and bottom-up strategies based on Fourier

transform ion cyclotron resonance (FTICR)-MS and LC separation of proteins[11, 12]. Kelleher *et al.* have developed a strategy for mapping intact protein isomers by combining 4 separation systems of isoelectric focusing (IEF), gel-eluted liquid fraction entrapment electrophoresis (GELFrEE), nano-LC and MS[13].

Even before the concept of proteomics, gel electrophoresis has been used for fractionation of complex cellular extract into protein spots visualized by staining[10]. High-resolution two-dimensional gel electrophoresis (2-D gel) combines isoelectric focusing (IEF) and SDS polyacrylamide gel electrophoresis[14, 15]. IEF is a technique developed through the 20th century by the efforts of many scientists, such as Vesterberg[16], Fawcett[17] and many others. Up to now, IEF electrophoresis has been the high-resolution technique of choice for separating amphoteric species according to their isoelectric points (p*I*) under the application of an electric field[18]. IEF electrophoresis can be realized with various platforms, including capillary electrophoresis (CE),[19] OFFGEL® electrophoresis[20] developed in our laboratory or simply gel electrophoresis using either a pH gradient gel or mobile ampholytes in a polyacrylamide gel[21]. After gel IEF, the biomolecules are either stained *e.g.* by Coomassie Brilliant Blue or silver and then extracted in a buffer for subsequent MS identification or LC-MS analysis, or are denatured for SDS electrophoresis[22]. In these protocols, procedures for in-gel digestion, chemical extraction and desalting are required, which are normally time-consuming and incomplete leading to sample loss, making the coupling of gel electrophoresis with MS a low throughput process difficult to automate. As a result, the 2D-gel-MS is less used than LC-MS in proteomics although 2D-gel is the high-resolution technique of choice for protein separation. It is very important to develop new ionisation techniques for directly coupling IEF-gel electrophoresis to MS in a highly automatic manner without the time-consuming steps of sample spot staining and chemical extraction of samples from the gel.

Ionisation is a key step in mass spectrometry to transform sample species into gas phase ions. This important step can be performed under vacuum or at atmospheric pressure. Most MALDI techniques are realised under vacuum. Recently, vacuum ionisation techniques have been developed by Trimpin *et al*, where the ionisation is mainly induced by the reduced pressure but not the high voltages nor a laser beam[23, 24]. The development of ambient ionisation is useful for fast analysis of untreated

samples. Classical ESI is the most widely used ambient ionisation technique; while other techniques such as desorption electrospray ionisation (DESI) developed by Cooks *et al*.[25] are extending the analytical applications. As described in the thesis of L. Qiao (thesis N° 5771), we have developed an electrostatic spray ionisation (ESTASI) method, which is also an ambient ionisation strategy, to *in-situ* generate molecular ions from untreated samples on an insulating surface[26].

In the present chapter, ESTASI is adapted to IEF gel electrophoresis, where charged samples in a gel were directly extracted by an electric field and then electro-sprayed for MS detection. According to our knowledge, it is the first attempt to *in-situ* ionise samples inside an IEF gel. As a result of the ionisation principle being similar to ESI, the limited tolerance to salts and SDS is still a challenge for extending the present ESTASI approach to other gel separation methods. Nevertheless, proof-of-concept results were obtained for ESTASI-MS analysis of proteins and peptides separated by IEF in immobilized pH gradient (IPG) gel with limits of detection (LOD) in the range of low nanograms, indicating a strategy for sample spots visualization that is sensitive, avoids chemical extraction procedures and provides molecular weight information at the same time. Since IEF gel electrophoresis is a high-resolution separation technique for proteins and especially protein isomers[27], the gel ESTASI-MS has potential application in top-down proteomics with the development of other techniques, such as gas phase fragmentation of large proteins, ultra-high resolution mass spectrometers and dedicated data processing software. Furthermore, since the ionisation was realised within an untreated gel after IEF, such strategy can be performed in a high throughput manner with the help of automatic positioning system.

2. Materials and methods

2.1 Materials

Immobilized pH gradient gels (Immobiline™ Dryplate pH 4 to 7) were purchased from Amersham Biosiences (Uppsala, Sweden). Cytochrome c from horse heart (12.3 kDa), myoglobin from horse skeletal muscle (17 kDa), bovine serum albumin (BSA, 66.4 kDa) and iodoacetamide (crystalline) were obtained from Sigma-Aldrich (Schnelldorf, Switzerland) as well as methanol and acetic acid of the purest grade (> 99.9%). Trypsin (bovine pancreas, 23.8 kDa) was from AppliChem GmbH (Darmstadt, Germany). Angiotensin I (NH_2−DRVYIHPFHL−COOH, 98%) was obtained from Bachem (Switzerland). 1,4-dithiothreitol (≥ 99%) was purchased from Fluka. Deionised water was purified by an alpha Q Millipore system (Zug, Switzerland) and used in all aqueous solutions.

2.2 BSA digestion

40 ml of 100 mM ammonium bicarbonate (NH_4HCO_3) was added to 100 ml of bovine serum albumin solution (87 mM). Afterwards, 5 ml of 200 mM 1,4-dithiothreitol (DTT) was added and the mixture was incubated at 95 °C for 5 min. 10 ml 200 mM iodoacetamide (IAA) was tacked and the solution was incubated for 20 min at room temperature in dark. Finally, trypsin was added with a final ratio of 1:30 (w/w, trypsin: protein), incubated at 37 °C for 20 h. The tryptic digests of BSA was stored under −20 °C.

2.3 Soluble *Escherichia coli* protein extract preparation

An overnight 200 ml culture of *Escherichia coli* (*E. coli*, strain DH5a) was collected by 10 min centrifugation at 5000 g and 4 °C. The cell pellet (0.6 g wet weight) was re-suspended in 3 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA) and cells were disrupted by sonication (10 x 10 pulses of 1 s at 30 W). Cell debris were removed by 10 min centrifugation at 2000 g. Ultracentrifugation (1 h at 100’000 g at 4°C) was applied to the total cell extract to remove membranes and membrane-bound proteins. The supernatant was collected and was considered as the soluble fraction of *E. coli* proteins.

2.4 Gel electrophoresis

The gel strips were cut from the Immobiline™ Dryplate pH 4 to 7 and then re-swelled in water for one hour before use. Depending on the application, gel strips with constant pH or pH gradient were prepared. After loading samples on the gel strips, electrophoresis was performed under different conditions according to the application. After electrophoresis, the gels were washed with water in order to remove any proteins staying on the surface of the gel before ESTASI-MS analysis.

For analysing cytochrome c, the electrophoresis was performed on a constant pH 4 gel strip under 300 V and 1 mA for 10 min with an EPS 3501 XL power supply (Amersham Pharmacia Biotech, Sweden). For analysing angiotensin I, the electrophoresis was performed on a gel strip with pH gradient 4 to 5.2 under the conditions of voltage applied = 500 V and limiting current = 100 mA for 30 min with the Agilent Fractionator 3100. For analysing BSA digests, IEF was performed by Agilent Fractionator 3100 on gel strips with pH gradient 4 to 7 under the condition of maximum current = 150 mA and voltage applied up to 4000 V until 10 kVh reached in 4 h. For analysing *E. coli* protein extract spiked with myoglobin and cytochrome c, IEF was performed with the following voltage program: 1 h at 500 V, then 1 h at 1000 V, and finally 5 h at 5000 V. The current limit was set at 150 mA. 400 mg of *E. coli* protein extract, 4 mg of myoglobin and 4 mg of cytochrome c were loaded on the gel.

2.5 Gel on a plastic support drilled with holes

The Immobiline™ Dryplate gel is commercially fixed on a 0.2 mm GelBond PAG film. Arrays of holes (1 mm in diameter) were drilled on the film by laser ablation through a metallic mask by a 193 nm ArF excimer laser beam (Lambda Physik, Göttingen, Germany, fluence = 0.2 J, frequency = 50 Hz). In total 2 lines of 10 holes were drilled on the film with the distance of 2 mm between each other. It is important to control the laser to just penetrate the plastic film while not burning the gel.

2.6 Gel-ESTASI-MS

Two strategies were used for IEF gel-ESTASI-MS. In the first strategy, droplets of an acidic buffer (50% methanol, 49% water and 1% acetic acid) were deposited manually on top of the gel that was fixed on a 0.2 mm GelBond PAG film. The ESTASI was performed as illustrated in Scheme 5.1. In the second strategy, ESTASI

was performed with the help of a plastic support drilled with holes. The gel was placed between two pieces of 0.2 mm GelBond PAG film, where one was drilled with holes by laser ablation. The holes were filled with the acidic buffer, and the ESTASI was performed as illustrated in Scheme 5.4.

In both cases, an electrode was placed behind the bottom plastic layer and the gel or part of the gel was placed close to the MS inlet to induce the electrostatic spray ionisation (Scheme 5.1 and 5.3). The electrode was connected with a DC high voltage (6.5 kV) source *via* switch 1 or grounded *via* switch 2. A special LabView programme was written to control the switches in order to synchronize their work. *Warning: Cables with ferrite bead should be used to avoid high frequency noise of HV*.

A linear ion trap mass spectrometer of Thermo LTQ Velos was used to detect the ions produced by ESTASI, where the MS inlet was always grounded. The spray voltage of the internal power source of the MS was set as 0. An enhanced ion trap scanning rate (10,000 amu/s) was used. For the analysis of BSA digest, the mass-to-charge ratios of peaks were read out to compare with the molecular weights of all the possible peptides generated from BSA by trypsin digestion. On-line tools of FindPept and FindMod from ExPASy (www.expasy.org) were used to help the comparison.

2.7 Gel based IEF-MALDI-MS

The IEF of BSA digest was performed using an IPG strip with same protocol described in Gel electrophoresis. After the IEF, the peptides were eluted from the IPG strip, using a Nanosep centrifugal device (Pall Life Sciences, Ann Arbor, MI, USA) following the protocol provided by the company (http://www.pall.com/main/Laboratory/Literature-Library-Details.page?id=2273).
Each extracted fraction was first completely lyophilised and then dissolved in 30 ml of water. 2 ml solution from each fraction was deposited on a MALDI plate and dried under ambient conditions. Afterwards, 1 ml matrix solution (10 mg/ml 2,5-dihydroxybenzoic acid in 50% methanol, 49% water and 1% acetic acid) was deposited on the sample and also dried under ambient conditions. The MALDI-MS analysis was performed on a Bruker MicroFlex LRF under reflectron positive ionisation mode.

2.8 OFFGEL IEF-ESI-MS

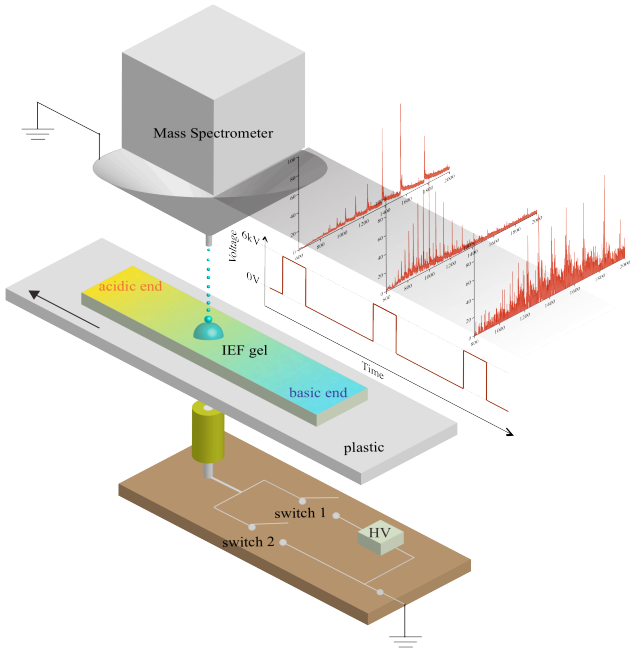
The Agilent Fractionator 3100 was used for OFFGEL separation of peptides. A 18-cm-long IPG gel strip with a liner pH gradient ranging from 4 to 7 was rehydrated in water for 1 hour. 50 ml of BSA digest (56 mM) was diluted with water to a final volume of 2.7 ml, and 150 ml of sample was loaded into each well (18 wells in total). The sample was then focused using the potential program with a maximum current of 50 mA, voltages ranging up to 8000 V until 64 kVh reached after 24 h. The recovered fractions (140 ml each) from each of the 18 wells were first lyophilised and then dissolved in 40 µl acidic buffer (50% methanol, 49% water and 1% acetic acid), respectively, for ESI-MS (Thermo LTQ velos) analysis. The standard commercial ESI source was used to produce ions from the solutions under infusion mode with a high voltage of 3.7 kV and a sample flow rate of 3 ml/min.

2.9 Numerical simulation

Numerical simulations were performed using finite element package COMSOL Multiphysics (version 3.5a) installed on a Mac Pro with four 2.66 GHz central processing units and 9.8 GB of RAM operating under Linux Ubuntu 8.04 platform. The electric field distribution in the polyacrylamide gel was simulated in a two-dimensional axis symmetry computational domain utilizing Laplace equation. For the finite element simulation, the relative permittivity values for air, water, and insulating plastic plate were taken from the Handbook of Chemistry and Physics (http://www.hbcpnetbase.com/). Other parameters used in the simulations were from the experiments, such as diameter of the hole drilled in the plastic cover (1 mm), the electrode diameter (2 mm), the insulating plate thickness (top and bottom, 0.2 mm) and the gel thickness (0.5 mm).

3. Results & Discussion

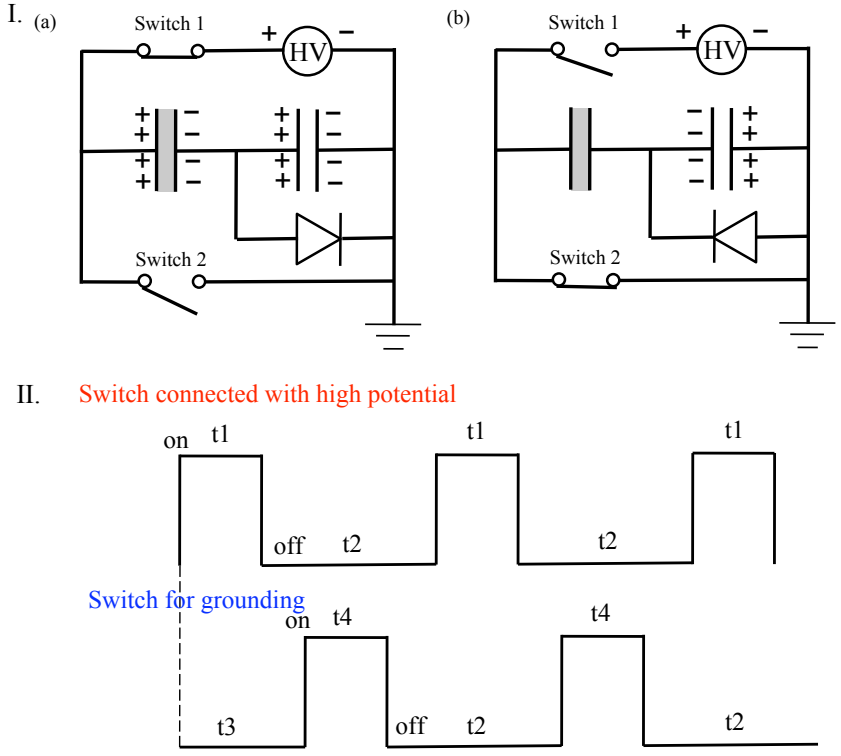
3.1 Electrostatic spray ionisation of peptide or protein from polyacrylamide gel



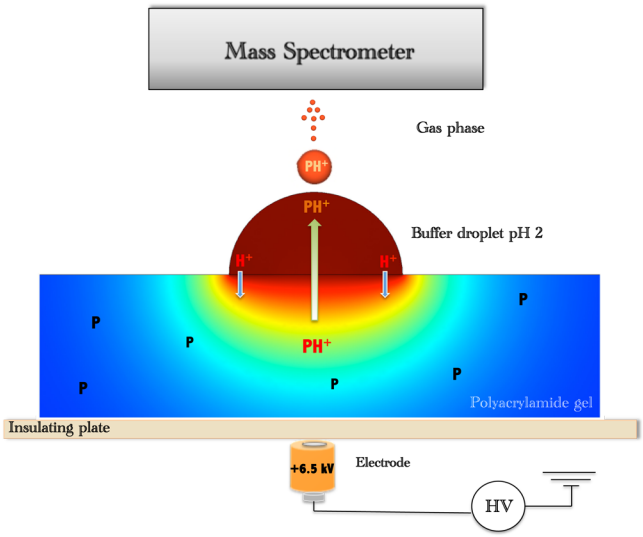
Scheme 5.1. Schematic representation of the electrostatic spray ionisation from a polyacrylamide gel, HV: high voltage.

The ESTASI-MS detection of samples inside polyacrylamide gel is schematically represented in Scheme 5.1. An immobilized pH gradient (IPG) strip containing fractions of proteins or peptides and supported on a thin piece of insulating polymer layer is placed in front of the MS inlet. An electrode is placed behind the plastic substrate and connected intermittently either to a direct current (DC) high voltage source or to ground using a pair of synchronised switches. A droplet of acidic buffer (49% water, 50% methanol and 1% acetic acid) is deposited on the gel between the electrode and the MS inlet. Upon application of a high voltage pulse, the droplet becomes polarised and as soon as the charge is large enough at the apex of the droplet for the electrostatic pressure to be larger than the Laplace pressure, a spray pulse of charged microdroplets occurs. When grounding the electrode again, a pulse spray of counter charges takes place to re-establish the electroneutrality of the droplet. For a positive high voltage, the cycle includes therefore first spray of cations and then of anions. By repeating this cycle at a given frequency, a series of pulsed sprays are

realized. This method is simple and can be used to analyse the separated sample components directly from a polyacrylamide gel without the need of extra processing steps. Electronic circuit and the Labview programme used to synchronise the two switches are shown in Scheme 5.2.



Scheme 5.2. I. Electronic circuit of electrostatic spray ionization during capacitors (a) charging and (b) discharging. The diode symbolises the direction of the spray current: spray of cations in (a) and spray of anions in (b). II. Schematic illustration of the performance of the two switches controlled by a Labview programme.



Scheme 5.3. Schematic representation of protein or peptide extraction from the polyacrylamide gel by applying high voltage, P: protein or peptide, PH^+: protonated protein or peptide. The colour gradient shows the diffusion of proton in a polyacrylamide gel obtained by finite element simulation with COSMOL Multiphysics.

To detect samples from a gel strip by ESTASI-MS, it is important to ensure that the analytes can be extracted into the droplets. In a first simple approach, acidic buffer droplets (pH = 2) were deposited by hand on an IPG strip with pH from 4 to 7. Because of the strong acidity of the droplets, peptides or proteins under the droplets became positively charged. When the positive high voltage was applied, these positively charged species were extracted into the droplets by the electric field as shown in Scheme 5.3. To guarantee an efficient extraction, different time sequences were tested to optimise the extraction-ionisation process. It was found that a long extraction time was preferable for acquiring good mass spectra and the optimised time sequence was set: t1 = t4 = 3 s, t2 = 9 s and t3 = 6 s (t1...4 are defined in Scheme 5.2 II).

With this simple approach based on the deposition of acidic droplets on top of the gel, the detection of cytochrome c in a gel strip using a linear ion trap mass spectrometer (Thermo LTQ Velos) was realized. Droplets (1 ml each) of cytochrome c solution with different concentrations (0.2 mg/ml and 0.02 mg/ml) were loaded on a constant pH 4 gel strips. An electrophoresis was performed for 10 min (300 V, 1 mA) with an EPS 3501 XL power supply (Amersham Pharmacia Biotech, Sweden) to migrate the sample and ensure that the sample has well penetrated within the gel. Afterwards, the gel was washed briefly with water in order to remove any proteins

present on the surface of the gel. Since cytochrome c is coloured, the protein band could be observed when the concentration was high (0.2 mg/ml), making the ESTASI-MS detection easy. When the concentration was lower, the gel was scanned by the ESTASI-MS to locate the protein band.

Figure 5.1 (a) shows one of the mass spectra of cytochrome c generated by ESTASI-MS, where the amount of protein in the band was at most 200 ng (16.3 pmol). Indeed, several pulses of ESTASI could happen when one droplet (1 ml) of the acidic buffer was deposited on the gel, resulting in several effective mass spectra obtained by the MS. The pulsed spray nature of ESTASI is beneficial to limit sample consumption.

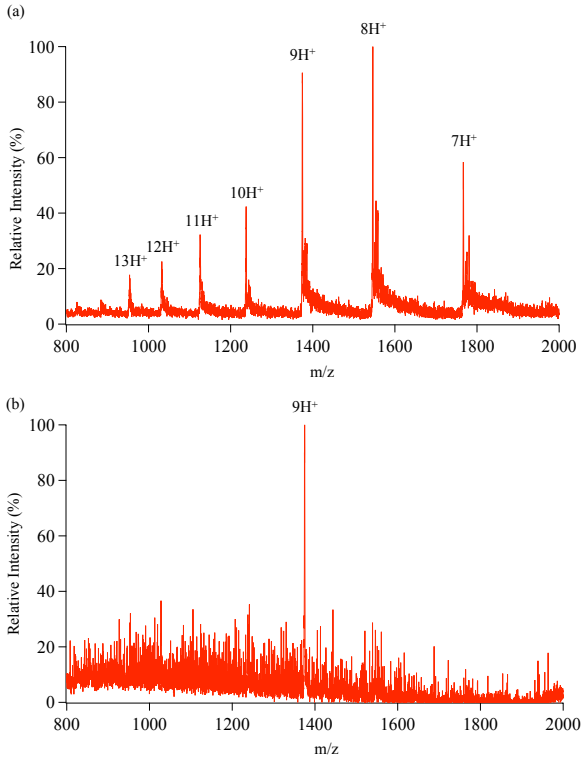


Figure 5.1. Mass spectra of cytochrome c with the amount of (a) 200 ng and (b) 20 ng in a polyacrylamide gel. The ions were generated by ESTASI when a pulsed positive high potential (6.5 kV) was applied to the electrode and when 1 ml of the acidic buffer was deposited on the gel.

When the amount of proteins in a band was too low, the concentration of proteins extracted into the droplet would also be too low to be detected by MS. With the current setup and a linear ion trap mass spectrometer (Thermo LTQ Velos), the limit of detection (LOD) of cytochrome c in a band was found to be 20 ng (1.63 pmol),

shown as Figure 5.1 (b). In practice, a higher sample amount is preferred since the observation of only one charge state is usually not sufficient for protein detection. There is a gap between the LOD of MS, which is normally in the low fmol range, and the in-gel ESTASI-MS strategy, which is in the low pmol range. However, the current gel profiling method is as good as or better than the classic Coomassie Brilliant Blue staining that normally needs 50-100 ng of protein in a band. With the direct ionisation of samples from gel by ESTASI, a maximum usage of the samples is guaranteed, and thereby further improvement of LOD and sensitivity can be expected by optimising the gel preparation and the ionisation device.

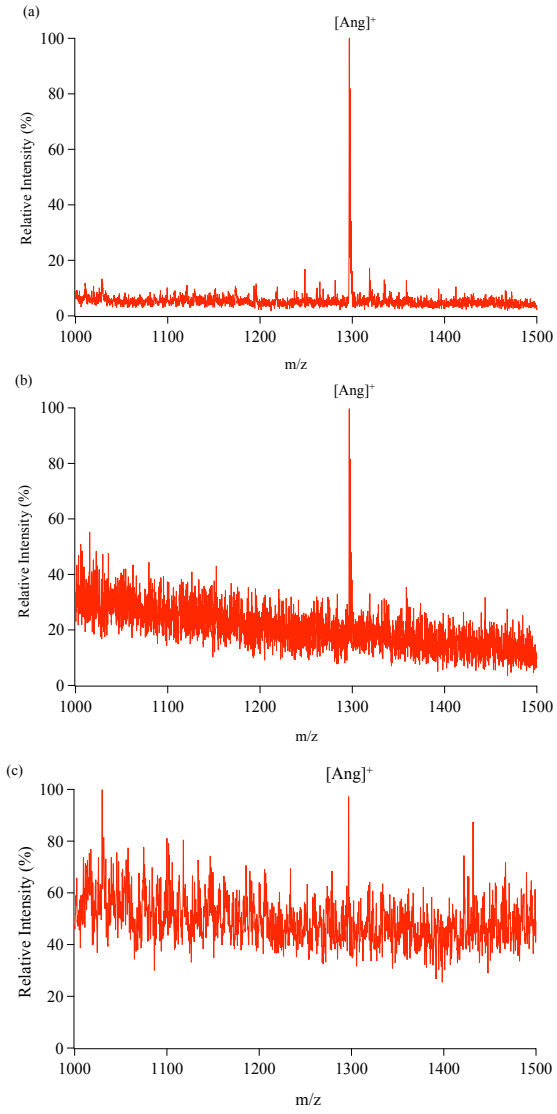
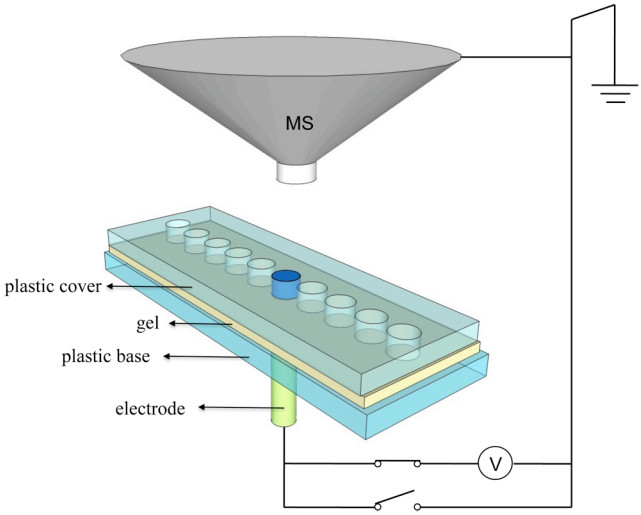


Figure 5.2. Mass spectra of angiotensin I with the amount of (a) 20 pmol, (b) 5 pmol and (c) 1 pmol in the gel with a pH range from 4 to 5.2 under positive MS mode. The ions were

generated by ESTASI when a pulsed positive high potential (6.5 kV) was applied to the electrode.

Angiotensin I peptide (NH_2-DRVYIHPFHL-COOH) was selected as a model sample to evaluate the ESTASI-MS detection of peptides in gel. 1 ml 20 mM angiotensin I solution was loaded on top of a gel strip with a pH range from 4 to 5.2. Gel electrophoresis was performed with the Agilent Fractionator 3100. Considering the isoeletric point (p*I*) of angiotensin I of 6.9, the peptide should migrate till the cathode after applying the voltage. Shown as Figure 5.2, the peptides were easily detected by ESTASI-MS after the electrophoresis by scanning the gel near the cathode area, where the amount of peptide in the band was 26 ng (20 pmol) in maximum. When the amount of angiotensin I was decreased to 6.5 ng (5 pmol), it was still easily detected by ESTASI-MS after IEF, Figure 5.2 (b). The LOD for ESTASI-MS in-gel detection of peptide was found to be 1.3 ng (1 pmol), Figure 5.2 (c), comparable to the LOD of protein by the in-gel ESTASI-MS.

3.2 Spatial resolution of ESTASI and IEF



Scheme 5.4. Schematic illustration of the ESTASI-MS analysis of proteins in gel when a plastic cover patterned with holes was used.

To go further towards practical applications, in-gel ESTASI was used to combine MS and IEF gel electrophoresis. It is then important for ESTASI to have a spatial resolution comparable to that of IEF. The spatial resolution of ESTASI is mainly determined by the electrode size and the droplet size. A plastic cover drilled with holes (1 mm in diameter) was placed on top of the gel as shown in Scheme 5.4. The

drilled holes can be filled with acidic buffer to control accurately the droplet size and thereby the spatial resolution of ESTASI. The holes can also be used for locating the spray position. As shown in Figure 5.3, ESTASI-MS worked well when a plastic cover patterned with holes was fixed on a gel containing cytochrome c.

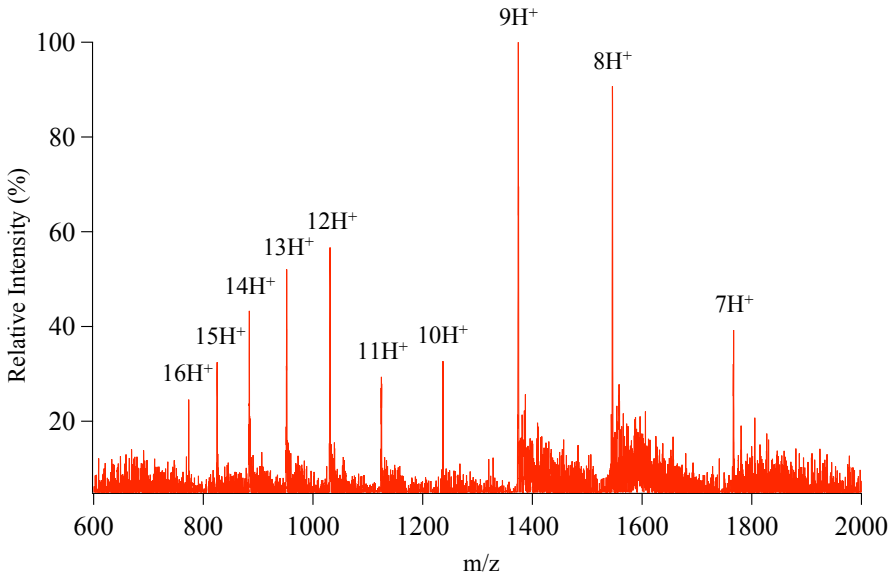


Figure 5.3. Mass spectrum of 200 ng cytochrome c in a polyacrylamide gel pH 4. The gel was under a plastic cover drilled with holes for filling acidic buffer. The ions were generated by ESTASI when a pulsed positive high potential (6.5 kV) was applied to the electrode. The label shows the charge states.

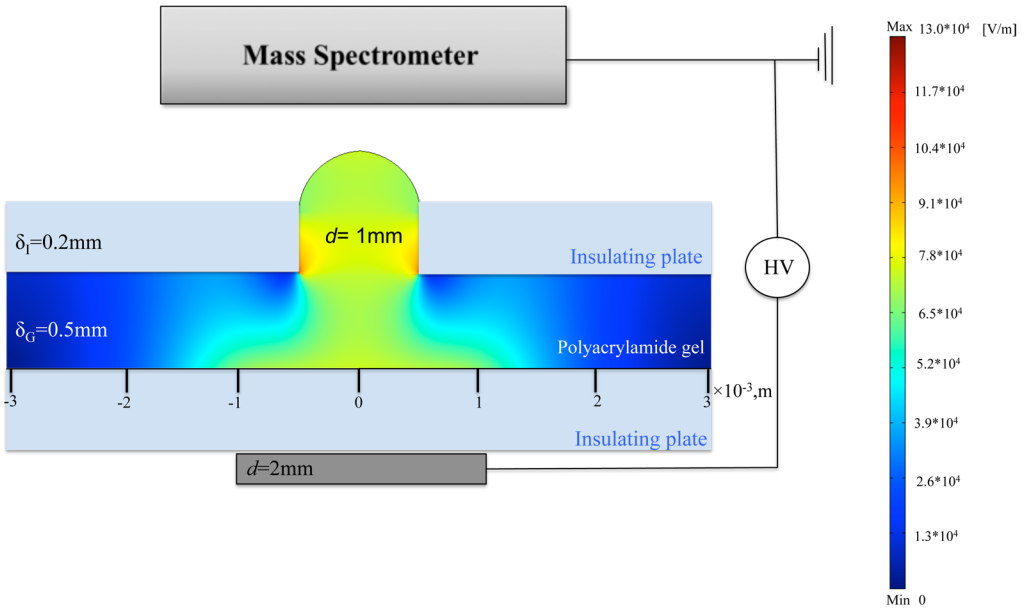


Figure 5.4. Finite element simulation of electric field in a polyacrylamide gel during sample extraction from the gel to the droplet.

The spatial resolution of ESTASI is mainly determined by the geometry of the cover and the electrode diameter. According to Scheme 5.3, charged proteins/peptides can be extracted from the gel to the droplet by electric field for ESTASI-MS detection. Therefore, the electric field in the gel determines the spatial resolution of ESTASI. Figure 5.4 represents the strong electric field in the gel given in V/m units. As it can be found from the calculations the spatial resolution is ~3 mm. Better resolution can be achieved by drilling smaller holes and using electrodes with smaller diameters, see Figure 5.5.

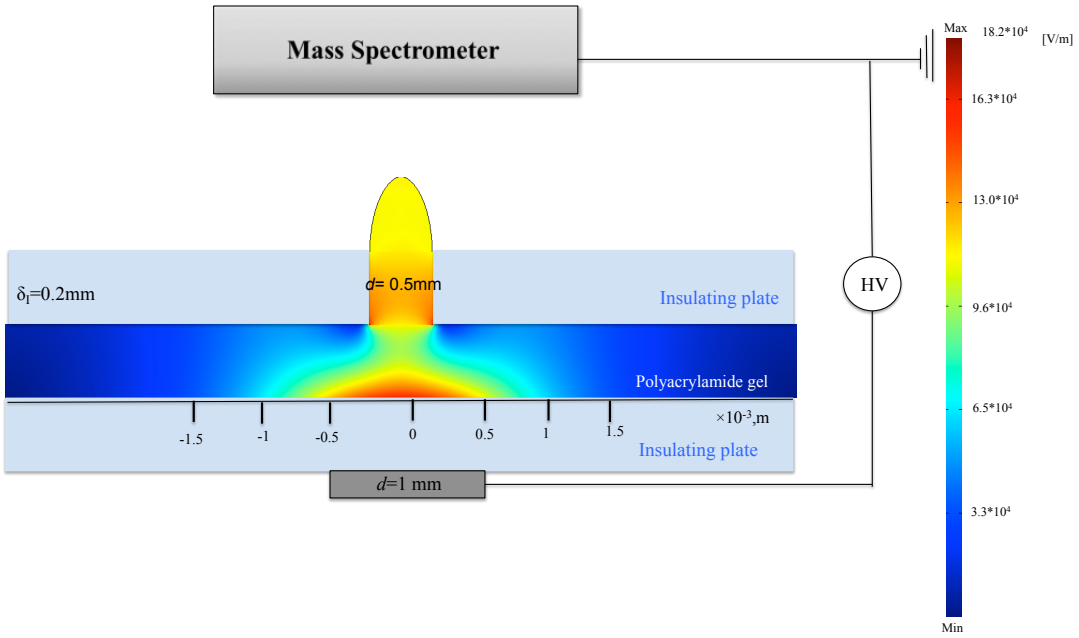


Figure 5.5. Finite element simulation of electric field in a polyacrylamide gel during sample extraction from the gel to the droplet. Comparing to Figure 3 in the manuscript, smaller electrode (1 mm in diameter) and holes (0.5 mm in diameter) are employed. Under this condition, the spatial resolution of gel ESTASI-MS is ~2 mm.

In IEF, the separation resolution is influenced by electric field strength, gel pH gradient and temperature. Normally, the buffer effect of the ampholyte molecules in gel generates a pH gradient with the resolution reaching 0.001 pH units, which allows the separation of proteins with p*I* differences of 0.02 pH units[28]. IPG strip with pH gradient of ~0.01 pH/mm was used in the current work. Therefore, the optimised spatial resolution of IEF separation was ~2 mm, comparable with that of ESTASI. As a result, it is possible to separately analyse samples fractionated into different bands by ESTASI-MS.

3.3 ESTASI-MS detection of a mixture of peptides separated by gel IEF

To demonstrate the feasibility of using ESTASI as an interface of gel IEF and MS, a mixture of peptides was separated by gel IEF and then detected by ESTASI-MS. A bovine serum albumin (BSA) tryptic digest sample was loaded on an IPG strip and separated by IEF electrophoresis. After separation, ESTASI was performed on different regions of the gel to analyse the separated peptides. The identification results from four droplets added onto different regions of the gel are shown in Figure 5.6 and Scheme 5.5, including an area close to the anode (pH = 4), an area with pH around 5.8, an area with pH around 6.2 and an area close to the cathode (pH = 7). 28, 13, 19 and 13 peptides were identified from the four areas, respectively, with good p*I* matching. Combining the results obtained from these 4 spots, the identification sequence coverage of BSA digest was found to be 74% (Scheme 5.5 and Tables 5.1.1-5.1.4). Indeed, several ESTASI-MS sprays were obtained from one droplet. Here, we show only one of several mass spectra obtained from the same droplet.

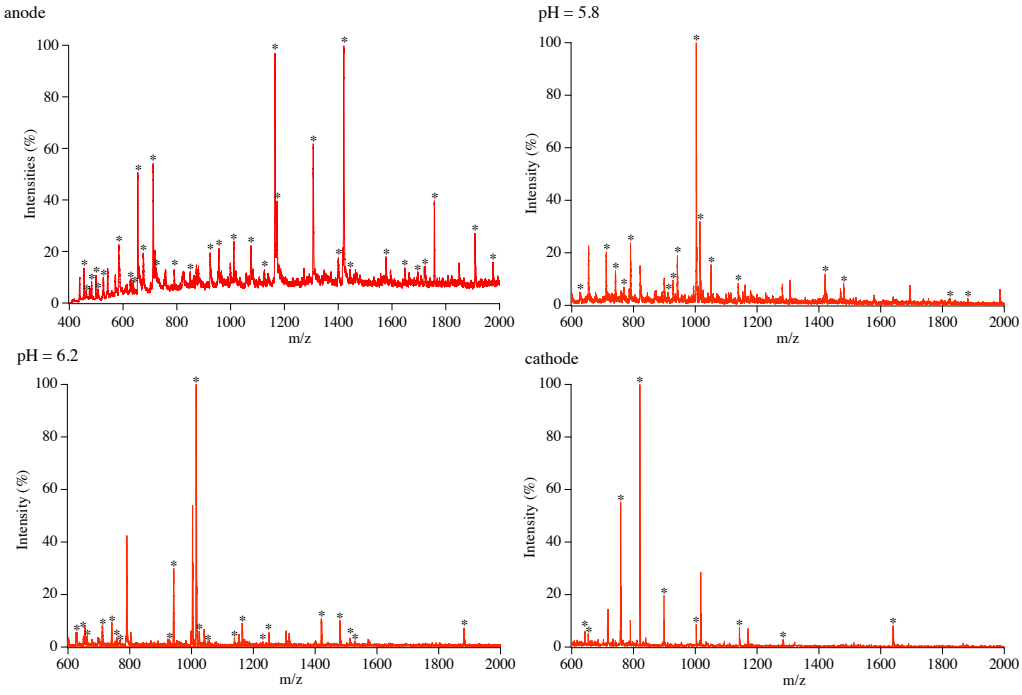


Figure 5.6. Mass spectra of BSA tryptic digest (5 ml, 56 mM) separated by IEF on an IPG strip under positive MS mode. The ions were generated by ESTASI from different areas of the gel. A pulsed positive high potential (6.5 kV) was applied to the electrode. The peaks may correspond to singly, doubly or triply charged ions.

MKWVTFISLLLLFSSAYSRGVFRRDTHKSEIAHRFKDLGEEHFKGLVLIAFSQYLQQCPFDEHV
KLVNELTEFAKTCVADESHAGCEKSLHTLFGDELCKVASLRETYGDMADCCEKQEPERNECF
LSHKDDSPDLPKLKPDPNTLCDEFKADEKKFWGKYLYEIARRHPYFYAPELLYYANKYNGVF
QECCQAEDKGACLLPKIETMREKVLASSARQRLRCASIQKFGERALKAWSVARLSQKFPKAEF
VEVTKLVTDLTKVHKECCHGDLLECADDRADLAKYICDNQDTISSKLKECCDKPLLEKSHCIA
EVEKDAIPENLPPLTADFAEDKDVCKNYQEAKDAFLGSFLYEYSRRHPEYAVSVLLRLAKEYE
ATLEECCAKDDPHACYSTVFDKLKHLVDEPQNLIKQNCDQFEKLGEYGFQNALIVRYTRKVP
QVSTPTLVEVSRSLGKVGTRCCTKPESERMPCTEDYLSLILNRLCVLHEKTPVSEKVTKCCTES
LVNRRPCFSALTPDETYVPKAFDEKLFTFHADICTLPDTEKQIKKQTALVELLKHKPKATEEQL
KTVMENFVAFVDKCCAADDKEACFAVEGPKLVVSTQTALA

Scheme 5.5. Sequence of bovine serum albumin. The amino acid residues in red colour were identified by the IEF-ESTASI-MS from 4 places of the gel (cathode, anode, pH = 5.8 and pH = 6.2). The identification sequence coverage from these 4 places was found as 74%.

Table 5.1.1 Peptides identified from BSA digest by in-gel IEF-ESTASI-MS from an area of the gel close to anode. PTMs: posttranslational modifications; Cys_CAM: carbamidomethylation cysteine; MSO: methionine sulfoxide; M.W.: monoisotopic molecular weight. The p*I* (isoelectric point) was calculated with the Compute p*I*/Mw tool on ExPASy (http://web.expasy.org/compute_pi/). The m/z is the monoisotopic peak value on the mass spectrum in figure 4 anode. These peaks can be singly, doubly or triply protonated ions.

Sequence	PTMs	M.W.	m/z	p*I*
AEFVEVTK		921.48	922.75	4.5
QNCDQFEK		1010.41	506.36, 1011.70	4.4
LVNELTEFAK		1162.62	1164.00	4.5
ETYGDMADCCEK		1363.47	455.83	3.9
YICDNQDTISSK		1385.61	463.12	4.2
TVMENFVAFVDK		1398.69	1400.00	4.4
ETYGDMADCCEK	Cys_CAM	1420.47	711.04, 1421.00	4.2
YICDNQDTISSK	Cys_CAM	1442.61	722.46, 1443.90	4.2
EYEATLEECCAK	Cys_CAM	1444.56	482.41	4.1
ETYGDMADCCEK	2Cys_CAM, MSO	1493.47	498.80	3.9
LKPDPNTLCDEFK	Cys_CAM	1575.74	526.34, 1577.00	4.6
ECCHGDLLECADDR		1577.59	789.97	4.1
ECCHGDLLECADDR	2Cys_CAM	1691.59	1693.00	4.1
YNGVFQECCQAEDK	2Cys_CAM	1746.65	583.31	4.1
CCAADDKEACFAVEGPK		1755.73	1757.00	4.3
VASLRETYGDMADCCEK		1889.80	630.78	4.3
LFTFHADICTLPDTEK	Cys_CAM	1906.89	636.73, 1908.1	4.5
LKPDPNTLCDEFKADEK		1961.94	655.04	4.4
VASLRETYGDMADCCEK	2Cys_CAM, MSO	2019.80	674.31	4.3
ECCHGDLLECADDRADLAK	3Cys_CAM	2246.87	1124.56	4.4
VASLRETYGDMADCCEKQEPER	MSO	2545.09	849.45	4.4
EYEATLEECCAKDDPHACYSTVFDK		2866.18	956.42	4.2
EYEATLEECCAKDDPHACYSTVFDKLK	2Cys_CAM	3221.36	1074.9	4.4
LAKEYEATLEECCAKDDPHACYSTVFDK	2Cys_CAM	3292.39	1647.39	4.4
ECCHGDLLECADDRADLAKYICDNQDTISSK		3443.47	1722.90	4.2
SHCIAEVEKDAIPENLPPLTADFAEDKDVCK	2Cys_CAM	3510.62	1171.33	4.3
ECCHGDLLECADDRADLAKYICDNQDTISSKLK	4Cys_CAM	3912.65	1305.30	4.4
ETYGDMADCCEKQEPERNECFLSHKDDSPDLPK	2Cys_CAM	3942.60	1972.50	4.3

Table 5.1.2 Peptides identified from BSA digest by in-gel ESTASI-MS from an area of the gel with pH around 5.8. PTMs: posttranslational modifications; Cys_CAM: carbamidomethylation cysteine; M.W.: monoisotopic molecular weight. The p*I* (isoelectric point) was calculated with the Compute p*I*/Mw tool on ExPASy (http://web.expasy.org/compute_pi/). The m/z is the monoisotopic peak value on the mass spectrum in figure 4 pH = 5.8. These peaks can be singly or doubly protonated ions. The peptides in this table were identified by several ESTASI-MS from the same place of the same gel, while the figure 4 pH = 5.8 shows only one of these mass spectra.

Sequence	PTMs	M.W.	m/z	p*I*
LVTDLTK		788.47	789.24	5.8
YLYEIAR		926.49	927.7	6.0
LVVSTQTALA		1001.58	1002.70	5.5
QTALVELLK		1013.62	1015.00	6.0
CCTKPESER		1051.45	1052.80	6.1
CCTESLVNR	2Cys_CAM	1137.45	1138.90	6.0
DVCKNYQEAK	Cys_CAM	1253.55	627.80	6.1
LKECCDKPLLEK		1417.74	710.00, 1419.10	6.2
LGEYGFQNALIVR		1478.80	740.52, 1480.10	6.0
LKECCDKPLLEK	2Cys_CAM	1531.78	767.04	6.2
YICDNQDTISSKLK		1626.80		6.0
RPCFSALTPDETYVPK		1822.90	912.16, 1824.05	6.1
RPCFSALTPDETYVPK	Cys_CAM	1879.92	941.24, 1881.10	6.1

Table 5.1.3 Peptides identified from BSA digest by in-gel ESTASI-MS from an area of the gel with pH around 6.2. PTMs: posttranslational modifications; Cys_CAM: carbamidomethylation cysteine; M.W.: monoisotopic molecular weight. The p*I* (isoelectric point) was calculated with the Compute p*I*/Mw tool on ExPASy (http://web.expasy.org/compute_pi/). The m/z is the monoisotopic peak value on the mass spectrum in figure 4 pH = 6.2. These peaks can be singly or doubly protonated ions. The peptides in this table were identified by several ESTASI-MS from the same place of the same gel, while the figure 4 pH = 6.2 show only one of these mass spectra.

Sequence	PTMs	M.W.	m/z	p*I*
IETMR		648.33	649.51	6.0
TPVSEK		659.36	660.52	5.7
YLYEIAR		926.49	927.36	6.0
QTALVELLK		1013.62	1014.80	6.0
CCTESLVNR		1023.46	1024.68	6.0
CCTKPESER		1051.45	1052.73	6.1
CCTKPESER	Cys_CAM	1108.47		6.1
CCTESLVNR	2Cys_CAM	1137.50	1138.61	6.0
CCTKPESER	2Cys_CAM	1165.49	1166.68	6.1
DVCKNYQEAK	Cys_CAM	1253.58	627.86, 1254.70	6.1
LKECCDKPLLEK		1417.74	710.00, 1418.90	6.2
LGEYGFQNALIVR		1478.80	740.50, 1480.00	6.0
VPQVSTPTLVEVSR		1510.84	756.49, 1512.11	6.0
LKECCDKPLLEK	2Cys_CAM	1531.78	767.01, 1532.93	6.2
YICDNQDTISSKLK	Cys_CAM	1683.82		6.0
DAFLGSFLYEYSRR		1722.84		6.1
RPCFSALTPDETYVPK		1822.90		6.1
RPCFSALTPDETYVPK	Cys_CAM	1879.92	941.08, 1881.13	6.1
NYQEAKDAFLGSFLYEYSRR		2456.18	1229.19	6.2

Table 5.1.4 Peptides identified from BSA digest by in-gel ESTASI-MS from an area of the gel close to cathode. PTMs: posttranslational modifications; Cys_CAM: carbamidomethylation cysteine; M.W.: monoisotopic molecular weight. The p*I* (isoelectric point) was calculated with the Compute p*I*/Mw tool on ExPASy (http://web.expasy.org/compute_pi/). The m/z is the monoisotopic peak value on the mass spectrum in figure 4 cathode. These peaks can be singly or doubly protonated ions. The peptides in this table were identified by several ESTASI-MS from the same place of the same gel, while the figure 4 cathode shows only one of these mass spectra.

Sequence	PTMs	M.W.	m/z	p*I*
DTHK		499.25		6.74
GACLLPK	Cys_CAM	757.42	758.61	8.22
LCVLHEK	Cys_CAM	897.48	898.65	6.74
ALKAWSVAR		1000.59	1001.73	11
GVFRRDTHK		1114.61		10.84
CASIQKFGER		1137.57		8.22
KQTALVELLK		1141.71	1142.90	8.59
QRLRCASIQK		1201.68		10.86
HPEYAVSVLLR		1282.71	642.5, 1283.90	6.75
HKPKATEEQLK		1307.73	954.95	8.51
QIKKQTALVELLK		1510.95		9.7
AWSVARLSQKFPK		1516.86		11.17
KVPQVSTPTLVEVSR		1638.94	820.6, 1640.10	8.75

For comparison, two parallel control experiments were performed, including OFFGEL IEF-ESI-MS and gel based IEF-MALDI-MS, which are standard methods used for peptide identification. In the case of OFFGEL IEF, samples were directly taken from the wells above the IPG strip. The ones close to the anode, above the areas with pH around 5.8 and 6.2, and close to the cathode were selected for ESI-MS identification (Thermo LTQ Velos). 12, 13, 10 and 5 peptides were identified from the four wells, respectively, and the identification sequence coverage summed from the four wells was found to be 48%, see Scheme 5.6 and Tables 5.2.1-5.2.4.

MKWVTFISLLLLFSSAYSRGVFRRDTHKSEIAHRFKDLGEEHFKGLVLIAFSQYLQQCPFDE
HVKLVNELTEFAKTCVADESHAGCEKSLHTLFGDELCKVASLRETYGDMADCCEKQEPERNE
CFLSHKDDSPDLPKLKPDPNTLCDEFKADEKKFWGKYLYEIARRHPYFYAPELLYYANKYNG
VFQECCQAEDKGACLLPKIETMREKVLASSARQRLRCASIQKFGERALKAWSVARLSQKFPK
AEFVEVTKLVTDLTKVHKECCHGDLLECADDRADLAKYICDNQDTISSKLKECCDKPLLEKSH
CIAEVEKDAIPENLPPLTADFAEDKDVCKNYQEAKDAFLGSFLYEYSRRHPEYAVSVLLRLAK
EYEATLEECCAKDDPHACYSTVFDKLKHLVDEPQNLIKQNCDQFEKLGEYGFQNALIVRYTR
KVPQVSTPTLVEVSRSLGKVGTRCCTKPESERMPCTEDYLSLILNRLCVLHEKTPVSEKVTKCC
TESLVNRRPCFSALTPDETYVPKAFDEKLFTFHADICTLPDTEKQIKKQTALVELLKHKPKATE
EQLKTVMENFVAFVDKCCAADDKEACFAVEGPKLVVSTQTALA

Scheme 5.6. Sequence of bovine serum albumin. The amino acid residues in red colour were identified by the OFFGEL IEF-ESI-MS from 4 places of the gel (cathode, anode, pH = 5.8 and pH = 6.2). The identification sequence coverage from these 4 places was found as 48%.

Table 5.2.1 Peptides identified from BSA digest by OFFGEL IEF-ESI-MS. The samples were taken from the well close to anode. PTMs: posttranslational modifications; Cys_CAM: carbamidomethylation cysteine; M.W.: monoisotopic molecular weight. The p*I* (isoelectric point) was calculated with the Compute p*I*/Mw tool on ExPASy (http://web.expasy.org/compute_pi/).

Sequence	PTMs	M.W.	p*I*
DDSPDLPK		885.42	3.9
QNCDQFEK		1010.42	4.4
EACFAVEGPK		1049.49	4.5
QNCDQFEK	Cys_CAM	1067.44	4.4
YICDNQDTISSK	Cys_CAM	1442.64	4.2
ETYGDMADCCEK	2Cys_CAM	1477.52	3.9
EYEATLEECCAK	2Cys_CAM	1501.61	4.1
YNGVFQECCQAEDK	2Cys_CAM	1746.71	4.1
DAIPENLPPLTADFAEDK		1954.96	3.8
TVMENFVAFVDKCCAADDK	Cys_CAM	2161.96	4.2
ECCHGDLLECADDRADLAK	3Cys_CAM	2246.94	4.2
DAIPENLPPLTADFAEDKDVCK	Cys_CAM	2457.18	4.0

Table 5.2.2 Peptides identified from BSA digest by OFFGEL IEF-ESI-MS. The samples were taken from the well with pH around 5.8. PTMs: posttranslational modifications; Cys_CAM: carbamidomethylation cysteine; M.W.: monoisotopic molecular weight. The p*I* (isoelectric point) was calculated with the Compute p*I*/Mw tool on ExPASy (http://web.expasy.org/compute_pi/).

Sequence	PTMs	M.W.	p*I*
IETMR		648.33	6.0
TPVSEK		659.36	5.7
NYQEAK		751.36	6.0
LVTDLTK		788.47	5.8
LVVSTQTALA		1001.58	5.5
SHCIAEVEK		1014.49	5.4
CCTESLVNR		1023.46	6.0
CCTESLVNR	2Cys_CAM	1137.50	6.0
CCTKPESER	2Cys_CAM	1165.49	6.1
FKDLGEEHFK		1248.62	5.5
LGEYGFQNALIVR		1478.80	6.0
VPQVSTPTLVEVSR		1510.84	6.0
RPCFSALTPDETYVPK	Cys_CAM	1879.92	6.1

Table 5.2.3 Peptides identified from BSA digest by OFFGEL IEF-ESI-MS. The samples were taken from the well with pH around 6.2. PTMs: posttranslational modifications; Cys_CAM: carbamidomethylation cysteine; M.W.: monoisotopic molecular weight. The p*I* (isoelectric point) was calculated with the Compute p*I*/Mw tool on ExPASy (http://web.expasy.org/compute_pi/).

Sequence	PTMs	M.W.	p*I*
ADLAK		516.30	5.9
IETMR		648.33	6.0
TPVSEK		659.36	5.8
LVTDLTK		788.47	5.8
CCTESLVNR		1023.46	6.0
DVCKNYQEAK	Cys_CAM	1253.58	6.1
LGEYGFQNALIVR		1478.80	6.0
VPQVSTPTLVEVSR		1510.84	6.0
LKECCDKPLLEK	2Cys_CAM	1531.78	6.2
RPCFSALTPDETYVPK	Cys_CAM	1879.92	6.1

Table 5.2.4 Peptides identified from BSA digest by OFFGEL IEF-ESI-MS. The samples were taken from the well close to cathode. PTMs: posttranslational modifications; Cys_CAM: carbamidomethylation cysteine; M.W.: monoisotopic molecular weight. The p*I* (isoelectric point) was calculated with the Compute p*I*/Mw tool on ExPASy (http://web.expasy.org/compute_pi/).

Sequence	PTMs	M.W.	p*I*
AWSVAR		688.37	9.8
SEIAHRFK		986.54	8.5
GVFRRDTHK		1114.61	10.8
DTHKSEIAHR		1192.60	6.9
VLASSARQRLR	Cys_CAM	1255.75	12.3

In the case of IEF-MALDI-MS, the IPG strip was cut into small pieces after IEF. The peptides were extracted from the gels, in the areas close to anode, with pH around 5.8 and 6.2, and close to cathode, for MALDI-MS analysis (Bruker MicroFlex LRF). 12, 9, 8 and 8 peptides were identified, respectively, and the identification sequence coverage summed from the four areas was found to be 47%, see Scheme 5.7 and Tables 5.3.1-5.3.4. These results clearly show that ESTASI provides an efficient coupling strategy between polyacrylamide gel IEF separation and MS identification. Considering the high sequence coverage, this method can be applied for protein structure characterisation in protein species proteomics. Besides, the combination of IEF with MS can provide information on peptide isoelectric point as well as peptide molecular weight, resulting in a highly confident identification even with low resolution mass spectrometer, such as ion trap, and without tandem MS[29].

MKWVTFISLLLLFSSAYSRGVFRRDTHKSEIAHRFKDLGEEHFKGLVLIAFSQYLQQCPFDEHV
KLVNELTEFAKTCVADESHAGCEKSLHTLFGDELCKVASLRETYGDMADCCEKQEPERNECF
LSHKDDSPDLPKLKPDPNTLCDEFKADEKKFWGKYLYEIARRHPYFYAPELLYYANKYNGVF
QECCQAEDKGACLLPKIETMREKVLASSARQRLRCASIQKFGERALKAWSVARLSQKFPKAEF
VEVTKLVTDLTKVHKECCHGDLLECADDRADLAKYICDNQDTISSKLKECCDKPLLEKSHCIA
EVEKDAIPENLPPLTADFAEDKDVCKNYQEAKDAFLGSFLYEYSRRHPEYAVSVLLRLAKEYE
ATLEECCAKDDPHACYSTVFDKLKHLVDEPQNLIKQNCDQFEKLGEYGFQNALIVRYTRKVP
QVSTPTLVEVSRSLGKVGTRCCTKPESERMPCTEDYLSLILNRLCVLHEKTPVSEKVTKCCTES
LVNRRPCFSALTPDETYVPKAFDEKLFTFHADICTLPDTEKQIKKQTALVELLKHKPKATEEQL
KTVMENFVAFVDKCCAADDKEACFAVEGPKLVVSTQTALA

Scheme 5.7. Sequence of bovine serum albumin. The amino acid residues in red colour were identified by the IEF-MALDI-MS from 4 places of the gel (cathode, anode, pH = 5.8 and pH = 6.2). The identification sequence coverage from these 4 places was found as 47%.

Table 5.3.1 Peptides identified from BSA digest by IEF-MALDI-MS from an area of the gel close to anode. PTMs: posttranslational modifications; Cys_CAM: carbamidomethylation cysteine; M.W.: monoisotopic molecular weight. The p*I* (isoelectric point) was calculated with the Compute p*I*/Mw tool on ExPASy (http://web.expasy.org/compute_pi/).

Sequence	PTMs	M.W.	p*I*
AFDEK		608.29	4.4
QEPER		657.32	4.5
ATEEQLK		817.43	4.5
DDSPDLPK		885.42	3.9
AEFVEVTK		921.49	4.5
QNCDQFEK	Cys_CAM	1067.44	4.4
LVNELTEFAK		1162.63	4.5
TVMENFVAFVDK		1398.69	4.4
YICDNQDTISSK	Cys_CAM	1442.64	4.2
EYEATLEECCAK	2Cys_CAM	1501.61	4.1
ECCHGDLLECADDR	3Cys_CAM	1748.66	4.1
LKPDPNTLCDEFKADEK	Cys_CAM	2018.97	4.4

Table 5.3.2 Peptides identified from BSA digest by IEF-MALDI-MS from an area of the gel around pH 5.8. PTMs: posttranslational modifications; Cys_CAM: carbamidomethylation cysteine; M.W.: monoisotopic molecular weight. The p*I* (isoelectric point) was calculated with the Compute p*I*/Mw tool on ExPASy (http://web.expasy.org/compute_pi/).

Sequence	PTMs	M.W.	p*I*
LVTDLTK		788.47	5.8
LVVSTQTALA		1001.58	5.5
QTALVELLK		1013.62	6.0
CCTESLVNR	2Cys_CAM	1137.49	6.0
FKDLGEEHFK		1248.62	5.5
LGEYGFQNALIVR		1478.79	6.0
VPQVSTPTLVEVSR		1510.84	6.0
LKECCDKPLLEK	2Cys_CAM	1531.78	6.2
RPCFSALTPDETYVPK	Cys_CAM	1879.92	6.1

Table 5.3.3 Peptides identified from BSA digest by IEF-MALDI-MS from an area of the gel around pH 6.2. PTMs: posttranslational modifications; Cys_CAM: carbamidomethylation cysteine; M.W.: monoisotopic molecular weight. The p*I* (isoelectric point) was calculated with the Compute p*I*/Mw tool on ExPASy (http://web.expasy.org/compute_pi/).

Sequence	PTMs	M.W.	p*I*
NYQEAK		751.36	6.0
QTALVELLK		1013.62	6.0
CCTESLVNR	2Cys_CAM	1137.49	6.0
CCTKPESER	2Cys_CAM	1165.49	6.1
LGEYGFQNALIVR		1478.79	6.0
VPQVSTPTLVEVSR		1510.84	6.0
LKECCDKPLLEK	2Cys_CAM	1531.78	6.2
RPCFSALTPDETYVPK	Cys_CAM	1879.92	6.1

Table 5.3.4 Peptides identified from BSA digest by IEF-MALDI-MS from an area of the gel around close to cathode. PTMs: posttranslational modifications; Cys_CAM: carbamidomethylation cysteine; M.W.: monoisotopic molecular weight. The p*I* (isoelectric point) was calculated with the Compute p*I*/Mw tool on ExPASy (http://web.expasy.org/compute_pi/).

Sequence	PTMs	M.W.	p*I*
LCVLHEK	Cys_CAM	897.48	6.7
NECFLSHK	Cys_CAM	1033.48	6.7
DTHKSEIAHR		1192.59	6.9
HPEYAVSVLLR		1282.70	6.8
RHPEYAVSVLLR		1438.80	8.8
ALKAWSVARLSQK		1456.85	11.2
QIKKQTALVELLK		1510.94	9.7
KVPQVSTPTLVEVSR		1638.93	8.8

3.4 ESTASI-MS in the analysis of protein mixtures separated by gel IEF

An important potential application of the IEF gel ESTASI-MS is direct analysis of proteins, which can be used in top-down proteomics in combination with other techniques, such as ultrahigh resolution mass spectrometer and gas phase fragmentation of intact proteins. In Figure 5.1, we have demonstrated that proteins can be directly ionised from a gel by ESTASI. However, with the current techniques available in our lab, it is hard to go further for the identification of unknown proteins, since only intact molecular weight of protein can be obtained yet not very accurate with the ion trap mass spectrometer.

To demonstrate the feasibility of ionising proteins from a complex background, we have applied this strategy to an *E. coli* protein extract spiked with myoglobin and cytochrome c. Shown as Figure 5.7, myoglobin and cytochrome c were identified from the complex protein mixture after IEF separation and ESTASI-MS. Mass spectra for *E.coli* extract were also obtained but the proteins identification could not be realised as mentioned before. Nevertheless, we have realized the *in-situ* ionisation of samples from the gel, which can be the beginning of an important development for direct coupling gel electrophoresis to MS, especially when combined with more advanced MS at the back end. In the current work, the gel ESTASI-MS is developed only based on polyacrylamide gel with constant pH or immobilized pH gradient.

Since the ESTASI holds a similar mechanism as electrospray ionisation,[26] it is largely influenced by the presence of salts and SDS. Indeed, detection of sample by ESTASI-MS from SDS-PAGE is quite difficult. The usage of salts, SDS and surfactants should be avoided during ESTASI-MS detection.

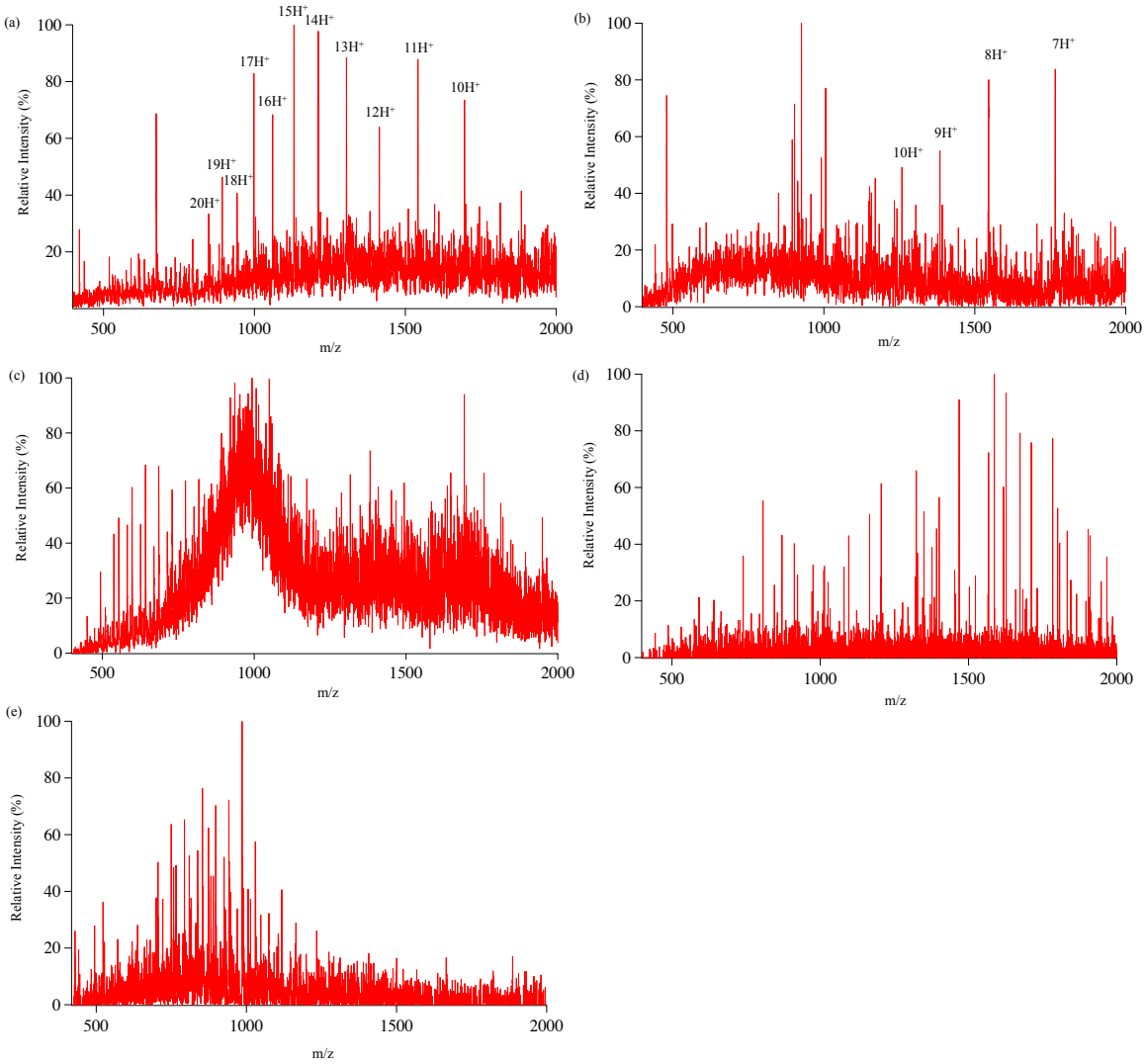


Figure 5.7. ESTASI-MS analysis of spiked *E.coli* extract samples separated by IEF: (a) fraction containing myoglobin, (b) fraction containing cytochrome c, (c) fraction at pH = 6.6, (d) fraction at pH = 5.1 and (e) fraction at pH = 4.4. The label shows the charge states.

4. Conclusions

A strategy to couple IEF gel electrophoresis with MS using electrostatic spray ionisation was developed. Proteins/peptides can be profiled after electrophoretic separation without extra processing steps. This direct coupling of ESTASI-MS with IEF gel separation improves the detection sensitivity compared with the classic Coomassie Brilliant Blue staining, with small amounts of target proteins or peptides as low as 20 ng or 1 ng in a band detectable, respectively. The strategy is also demonstrated as a more powerful method to couple gel IEF with MS than the developed standard methods. With these proof of concept results, we could expect the ESTASI-MS method to be applied in a variety of research fields, not only in the profiling of gels but also the imaging of chemicals on any surface.

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Chapter VI.

Electrostatic Spray Ionization Mass Spectrometry Sniffing for perfume fingerprinting

Based on E. Tobolkina et al. Rapid Communications in Mass Spectrometry, 2013, 27 (21), 2310–2316.

1. Introduction

Mass spectrometry has been one of the most rapidly developing chemical analysis techniques over the last decades, applied in various research fields, including organic chemistry, polymer science, life science, *etc*. One major challenge in mass spectrometry is to directly analyze untreated samples in their ambient state, which calls the development of new atmospheric ionization methods[1]. Nowadays, techniques, such as electrospray ionization mass spectrometry (ESI-MS),[2, 3] desorption electrospray ionization (DESI)[1] MS and extractive electrospray ionization (EESI)[4] MS, allow to identify and classify liquid samples in a rapid online mode[5].

As previously described in Chapter V, we have developed an electrostatic spray ionization (ESTASI) method, which can generate protonated molecules from samples deposited as droplets on a plastic substrate or inside a polyacrylamide gel[6, 7]. Herein, the ESTASI is further applied on a piece of lint-free paper. Indeed, a paper spray ionization method was introduced by Wang *et al.* in 2010, where samples were deposited on a piece of paper that was connected with high voltage for electrospray ionization[8]. Such paper spray ionization has been applied for qualitative and quantitative analysis of dried biofluid spots and drugs.

Different from the previously reported work spray ionization approaches, the high voltage (HV) is not applied directly to the paper but on an electrode that is isolated from the paper by a plastic plate (insulator) during paper ESTASI. The electrode is connected in turn to the HV and the ground under the control of two switches to induce ESTASI under ambient conditions. When the electrode is connected to HV, spray of cations happens during charging a capacitor formed by electrode, insulator and sample solution. When the electrode is ground, spray of anions happens during discharging the capacitor. A modified "sniffing" transfer capillary collects the generated ions to MS for analysis. The paper ESTASI is here used for protein/peptide analysis and fast characterization of perfumes that are deposited or sprayed on a piece of lint-free paper.

Fine fragrances are mixtures of volatile synthetic chemicals as well as natural extracts such as essential oil and concentrates of flower, fruits, spices, *etc* obtained by a wide range of methods like expression, solvent extract and steam distillation[9]. Perfumes consist of typically 10 to 300 of such constituents, leading to very complex mixtures[10]. Nevertheless, thanks to the constant improvement in terms of speed and

efficiency of analytical techniques[11], it is nowadays possible to analyze the composition of perfumes, leading to the risk of counterfeit for all major brands of perfume. Such imitative fragrances are usually of lower quality than the authentic ones, and represent a violation of the Intellectual Rights, creating a possible threat for perfume companies. Therefore, it is important to develop efficient chemical analysis techniques to screen rapidly perfume samples[12].

Gas chromatography (GC) coupled with the detection methods, such as flame ionization detection (FID)[13], Fourier transform infrared spectroscopy (FT-IR), mass spectrometry (MS)[14-17] and tandem mass spectrometry (MS/MS)[18], has been the techniques of choice for qualitative and quantitative analyses of volatile and semi-volatile compounds in cosmetic products and perfume[19]. However, these widely used methods suffer from some limitations as follow: (i) sample preparation complexity in particular with the cosmetics containing also non-volatiles ingredients not compatible with GC-MS analysis, (ii) time of the analysis which prevents the control of large set of samples[20]. To circumvent these limitations, the development of new analytical methods for the screening of fragrances is needed.

Recently, EESI-MS was applied for the classification of perfume as a high throughput and sample-preparation-free method[4]. ESI-MS in a standard infusion mode can also be applied in perfume analysis, but limited by the preparation procedures because of the strong adsorption of volatile molecules inside the syringe and the transfer capillary. Herein, we have demonstrated that paper ESTASI-MS is a technique that generates perfume mass spectra similar to those obtained using an electrospray ionization (ESI) source but without sample preparation, thereby in a more convenient and faster manner. Six authentic fragrances and a model perfume prepared by mixing 10 different compounds were used as samples for analysis. To assess the performance of this new experimental setup, analyses of peptides and proteins deposited on paper were first carried out.

2. Materials and methods

2.1 Materials

Angiotensin I (NH_2−DRVYIHPFHL−COOH, 98%) was obtained from Bachem (Basel, Switzerland). Cytochrome c from horse heart (12.3 kDa) as well as methanol and acetic acid of the purest grade (> 99.9%) were obtained from Sigma-Aldrich (Schnelldorf, Switzerland). Deionized (DI) water was purified by an alpha Q Millipore system (Zug, Switzerland).

A "model perfume" sample consisting of ten different compounds (ambrox, citronellyl nitrile, cyclogalbanate, damascenone, diphenyl ether, hedione, neobutenone alpha, iso e super, sclareolate, tonalide pf) was prepared in our laboratory. The structure and molecular weight of these compounds are presented in *Appendix 6.0*. Six authentic fragrances: ´Un Jardin sur le Nil´ by Hermes, ´Flower´ by Kenzo, ´Ange ou demon´ by Givenchy, ´Encre Noire´ by Lalique, ´Midnight in Paris´ by Van Cleef & Arpels and ´The one gentleman´ by Dolce & Gabbana, as well as the perfume testing strip and "Chanel" blotting paper were obtained from local stores. Lint free paper, Contec Wipe Nw CEL/POLY 9X9IN PK300 C1-99, was acquired from Contec (Toledo, USA).

2.2 Paper electrostatic-spray ionization

5 μL of the sample containing either the peptide/protein in the acidic buffer (50% methanol, 49% water and 1% acetic acid) or the perfume sample was deposited with a micropipette or nebulized directly from the perfume bottle on top of the paper strip (6 cm long, 2 cm wide), which was placed under the modified mass spectrometer inlet. The paper strip here can be the fragrance testing strip, the "Chanel" blotting paper or the lint-free paper. An electrode was placed behind and isolated from the paper by a piece of plastic, and facing the sample to induce electrostatic-spray. The electrode was connected either to a direct current (DC) high voltage (9 kV) source or to ground using two switches. A special LabView programme was written to control the switches in order to synchronize their work[6].

2.3 Mass spectrometer

A linear ion trap mass spectrometer of Thermo LTQ Velos (Reinach, Switzerland) was used to detect the ions produced by ESTASI with a modified extended transfer capillary for "sniffing" ions from the paper. Electrospray voltage of the LTQ Velos was set as 0. A high voltage battery as an external power source was used for the ESTASI. An enhanced ion trap scanning rate (10,000 *m/z* units/s) was used for the MS analysis. All the spectra were recorded in positive ion mode.

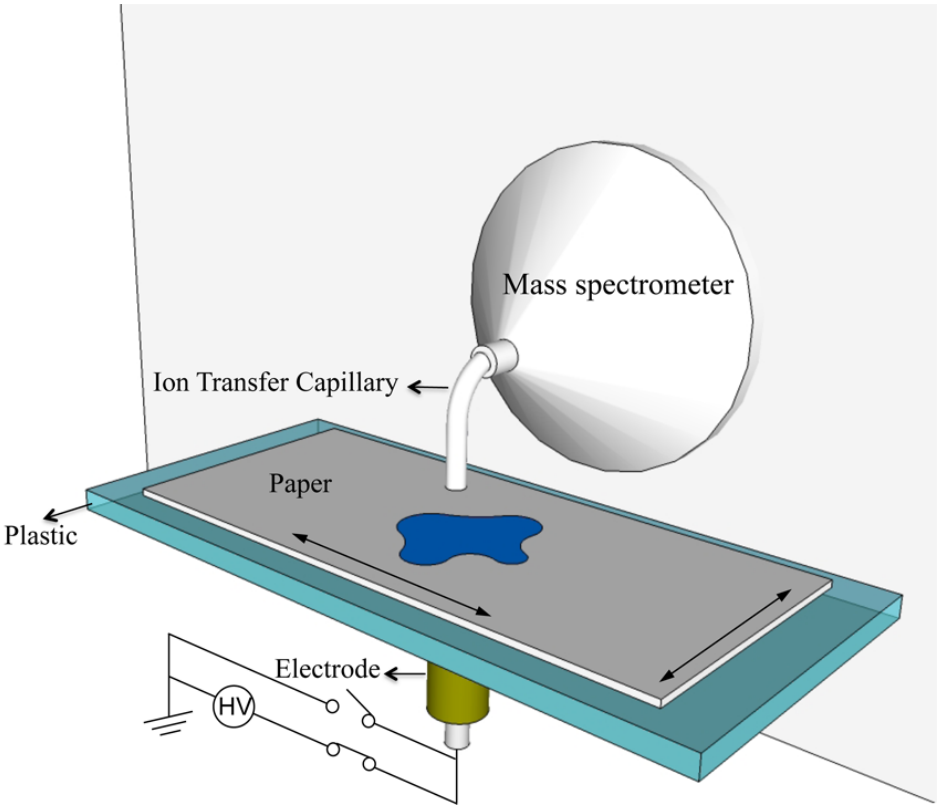
3. Results and Discussion

3.1 Paper electrostatic spray ionization mass spectrometry

To characterize the perfume from the lint-free paper, the electrostatic spray ionization (ESTASI) method was used. The principle of the technique was discussed and explained in Chapter V[6, 7]. In simple words, a sample solution is placed on an inert insulating support placed in front of the mass spectrometer inlet. An electrode is placed behind the support and is connected to a pulsed high voltage source. When a positive high voltage is applied, charge separation happens in the sample solution, and the sample-air interface becomes polarized. When the charge accumulated is large enough for the electrostatic pressure to be larger than the Laplace pressure, electrospray of cations towards the mass spectrometer inlet happens, leaving net negative charges in the sample solution. By grounding the electrode, anions will charge again the liquid-air interface, leading to electrospray of anions.

ESTASI of a sample solution from a microchannel, a capillary, and droplets on a plastic plate was reported[6]. Here, we demonstrate that the ESTASI can also be used for samples within a piece of lint-free paper that is placed on a plastic plate, as shown in Scheme 6.1. The sample solution was deposited on top of the paper and adsorbed by the fibers of the paper quickly without forming droplets or only forming a thin layer of liquid. The paper strip was placed on the insulating plate between the electrode and mass spectrometer inlet, where the plate can move in *x,y* directions in order to set the sample right under and close enough to the mass spectrometer inlet. A high potential of 9.0 kV was used to induce ESTASI. By applying a pulsed high voltage to the electrode, samples were ionized for MS detection before the solvent was completely evaporated from the paper. The ion transfer capillary of mass

spectrometer was modified as shown in Scheme 6.1 to "sniff" the ions from the paper, which was done for conveniently scanning a horizontal surface plate placed in front the Thermo LTQ Velos.



Scheme 6.1. Schematic representation of the paper ESTASI to MS with modified ion transfer capillary.

To test this new experimental setup, different experiments were first carried out on model samples. First, paper ESTASI was performed with the angiotensin I peptide (NH_2-DRVYIHPFHL-COOH) at different concentrations in an acid buffer (50% methanol, 49% water and 1% acetic acid). The sample (5 μL) was deposited on top of the testing strip and the high voltage of 9.0 kV was used for ESTASI. During ESTASI, no droplets were formed. The spray happened either from a thin and flat layer of liquid or from the solution in the structure of the paper that can be viewed as arrays of micropores. As soon as the positive high voltage was applied on the electrode, the cations would be generated by ESTASI for MS detection. As shown in Figure 6.1, the peptide was easily detected by paper ESTASI-MS and the limits of detection (LOD) of the peptide was found as ~250 nM. In a similar way, the protein

cytochrome c in ESI buffer was also analysed by the paper ESTASI-MS. As shown in Figure 6.1, this protein can be easily detected with the LOD of 1.6 μM.

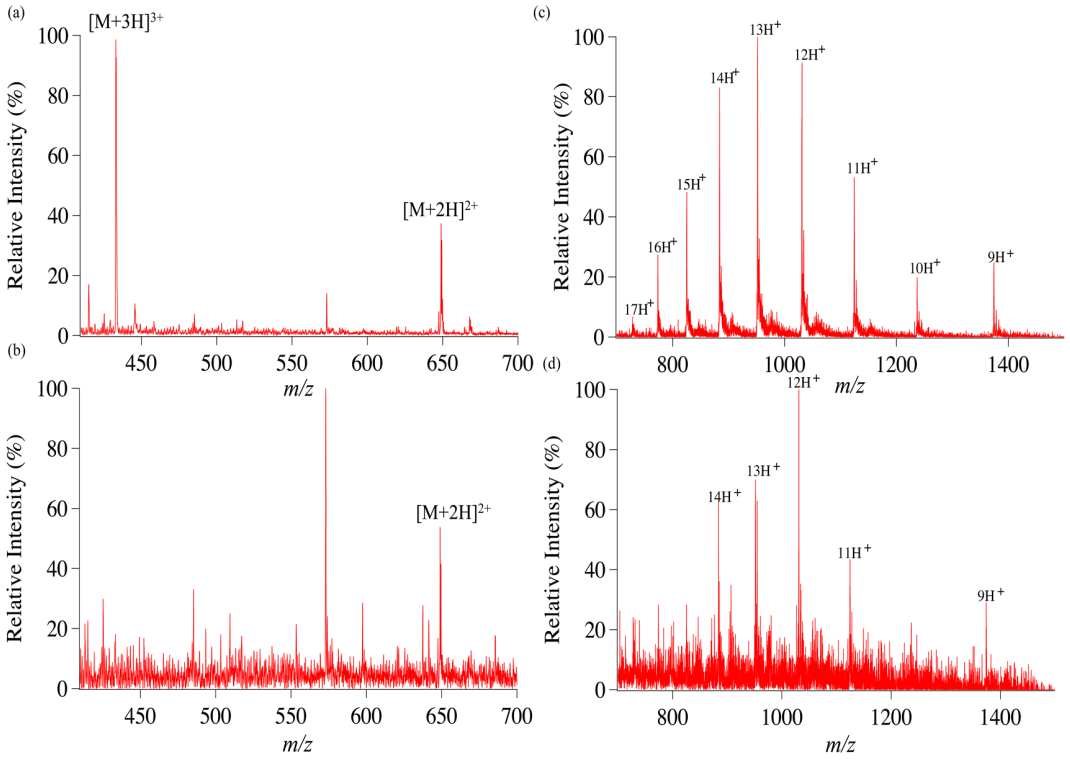


Figure 6.1. Mass spectra of: (a) 25 μM of angiotensin I, (b) 250 nM of angiotensin I, (c) 16 μM of cytochrome c, and (d) 1.6 μM of cytochrome C sprayed from the paper strip by ESTASI. All the mass spectra were taken under positive MS mode. The ions were generated by paper ESTASI when a pulsed positive high potential (9.0 kV) was applied to the electrode.

3.2 Paper ESTASI for the analysis of a model perfume

The perfume sample may contain hundreds of different constituents. It is quite difficult to detect all of them using mass spectrometry, as not all the compounds from the sample can be ionized by electrospray ionization. A "model perfume" sample was used as a standard sample to demonstrate the concept of using paper ESTASI-MS for perfume analysis. The model perfume was prepared by mixing 10 compounds in different ratios.

The model perfume was firstly analyzed by ESI-MS using the commercially equipped ESI source under direct-infusion and positive ion mode. The mass spectrum obtained is shown as Figure 6.2a 5 out of 10 compounds from the mixture were

detected and identified, while the others cannot be ionized by ESI to be detected. There are peaks on the mass spectrum that cannot be identified as compounds of the model perfume and may be attributed to products of chemical reactions or electrochemical reactions during the ESI or as the background peaks of the instrument. Although good spectrum for the model perfume was obtained by the ESI with commercially equipped ESI source under direct-infusion mode, it is not an ideal technique for high throughput analysis of perfume. Indeed, the volatile compounds in the perfume are easily adsorbed as contaminants on the wall of the polymer capillary that is used to transfer sample solution from syringe to the ESI source. It took hours of washing of the capillary to remove completely the adsorbed molecules to recover a clean background.

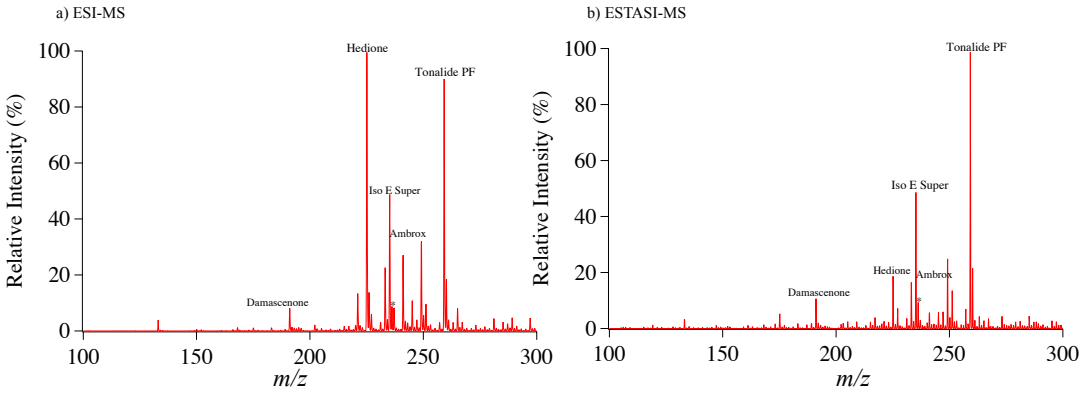


Figure 6.2. Mass spectra of the model perfume sample obtained by **a)** ESI-MS and **b)** ESTASI-MS in a positive ion mode. The labeled peaks correspond to singly protonated molecules.

The paper ESTASI-MS was used to avoid this cleaning step. The model perfume was deposited with a micropipette on top of the lint-free paper just under MS inlet. By applying the high voltage (9 kV), the sample was sprayed from the surface of the paper and directly analyzed by MS in a positive ion mode. As shown in Figure 6.2b, the same 5 compounds were identified by paper ESTASI-MS. Since the lint-free paper strip is disposable, the cleaning steps are avoided and no contamination between strip-to-strip was observable. Negative ion mode was also tested, but did not give any identification of the perfume compounds with both ESI-MS and ESTASI-MS, because none of them can be easily deprotonated.

3.3 Paper ESTASI for the analysis of six authentic fragrances

The paper ESTASI was further applied for the fast analysis of real complex perfume samples. Six authentic fragrances including three women's and three men's were analyzed. Each fragrance was deposited (5 μL) by a micropipette or nebulized on top of a paper strip, which is then placed under the ion transfer capillary. ESTASI was performed from the region where the sample was loaded and the compounds of the perfume were extracted and detected by MS. Figure 6.3 shows the paper ESTASI-MS results of three women's perfume fragrances ("Givenchy", "Hermes", "Kenzo"), which were obtained with the different types of paper strips. ESI-MS was also performed for all of the fragrances with the commercial source mounted on the LTQ Velos for comparison.

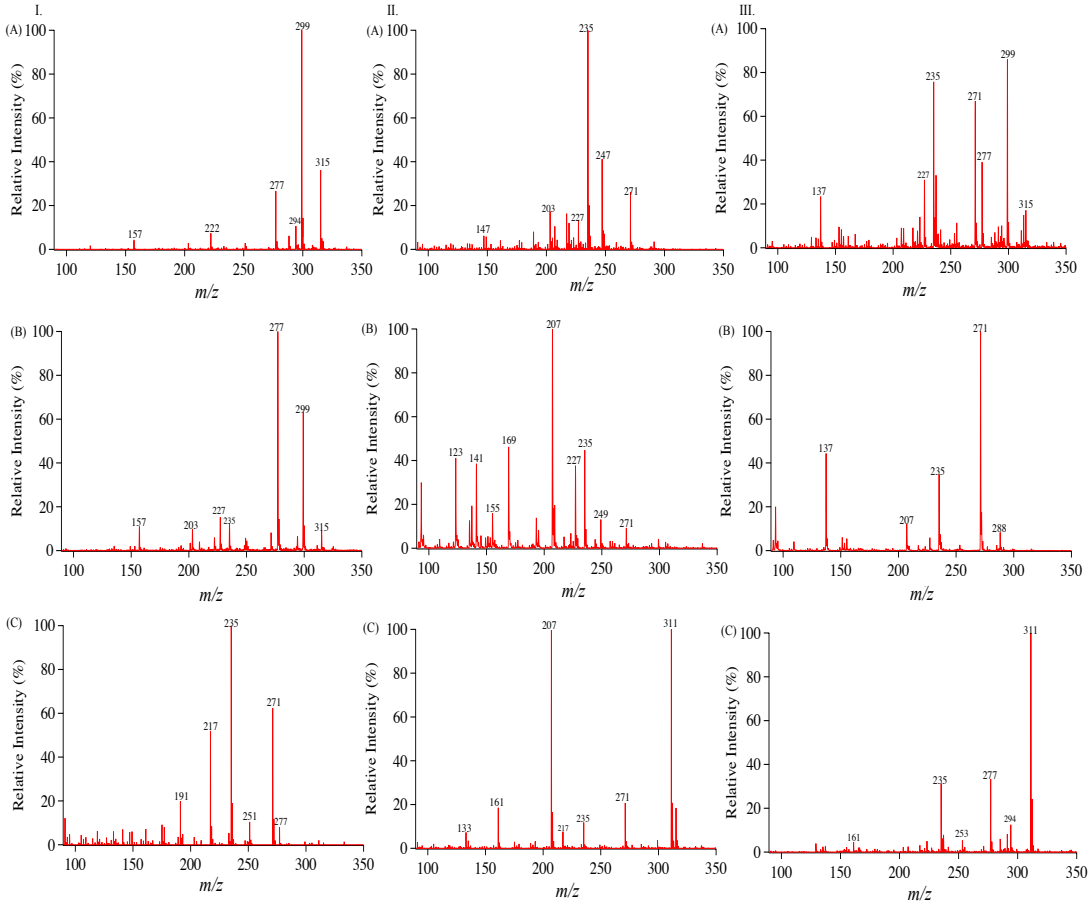


Figure 6.3. Mass spectra of three authentic fragrances (I. Ange ou demon´ by Givenchy; II. ´Un Jardin sur le Nil´by Hermes; III. ´Flower´ by Kenzo) obtained by (A) ESI with commercially equipped ESI source under direct-infusion mode, (B) paper ESTASI-MS with the substrate of lint-free paper and (C) paper ESTASI-MS with the substrate of perfume testing strip from the local shop. The MS experiments were performed in a positive ion mode.

It can be clearly seen that the most abundant peaks appear in the *m/z* 100-300 range, corresponding to highly volatile compounds. The results obtained by ESI-MS of each fragrance were compared with those obtained from paper ESTASI-MS with lint-free or perfume testing strip. Depending on the spray surface, the abundance of the peaks of different ions detected is different. This could be induced by the fact that the adsorption of the perfume compounds by different types of paper is different (see Figures 6.3 and 6.5). As shown in Figure 6.3I and Table 6.1, ESTASI-MS from lint-free paper gave the most similar observation of peaks from the "Givenchy" perfume as the ESI-MS. The peaks with *m/z* 157, 222, 277, 299 and 315 were abundant and observed by both the ESI and paper ESTASI with lint-free paper. However, the paper ESTASI of this perfume from perfume testing strip taken from the perfume shop as well showed a result similar for those obtained from ESI-MS.

Before the analyses of the commercial fragrances from the paper, blank analysis was performed by ESTASI-MS. Several microliters of ESI buffer were deposited on the paper strip and the high voltage was applied. It was proven that none of the peaks detected from the perfume fragrances was observed during the "blank" experiment.

Figure 6.4 represents the steps of depositing and analyzing the "Givenchy" fragrance from the blotting paper by ESTASI-MS. The sample was firstly nebulized on the skin from the original bottle and then soaked by the blotting paper, which was afterwards placed on the insulating plate for ESTASI-MS analysis. As shown in Figure 6.4 and Table 6.1, the blotting method also gave a detection of compounds similar as the ESI-MS.

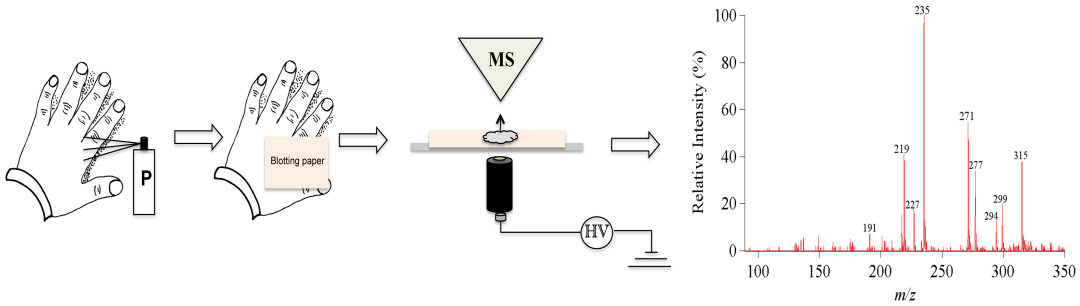


Figure 6.4. "Givenchy" perfume nebulization on the hand, soaking the perfume with a blotting paper from the hand and the paper ESTASI-MS analysis, P: perfume, MS: mass spectrometry analysis.

Shown as Figure 6.3II and III, the compounds detected by ESI-MS and paper ESTASI-MS with different substrates share some similarities but show also some differences. With the current methods, of course it is not possible to identify each

observed peak and it is not possible to observe most compounds from each perfume. However, it is possible to use the spectra as chemical fingerprints for the perfumes since the spectra between different perfumes are highly different and the spectra for each perfume are reproducible. Combining the spectra obtained with different paper substrates, such chemical fingerprints for perfumes can be good identities.

Table 6.1. Peak list of the ESI-MS and ESTASI-MS with the lint free, the blotting and the fragrance testing strip of the "Givenchy" perfume (+ observed; - not observed).

m/z	ESI-MS	Lint-free paper	Testing strip	Blotting paper
157	+	+	-	-
161	-	-	+	-
175	-	-	+	-
191	-	-	+	+
203	+	+	-	-
209	-	+	-	-
222	+	+	+	+
227	-	+	-	-
235	-	+	+	+
251	+	+	+	-
271	-	+	+	+
277	+	+	+	+
294	+	+	-	-
299	+	+	-	+
315	+	+	-	+

Three men's perfumes were also analyzed and compared by ESI-MS, paper ESTASI-MS with lint-free paper and paper ESTASI-MS with fragrance testing strip. As shown in Figure 6.5, the most abundant peaks also appear in the *m/z* 100-300 ranges, corresponding to volatile compounds of the perfume that could be easily desorbed from the surface of the paper strip. The spectra between perfumes obtained with the same method looks different, further suggesting these spectra as chemical fingerprints of perfumes.

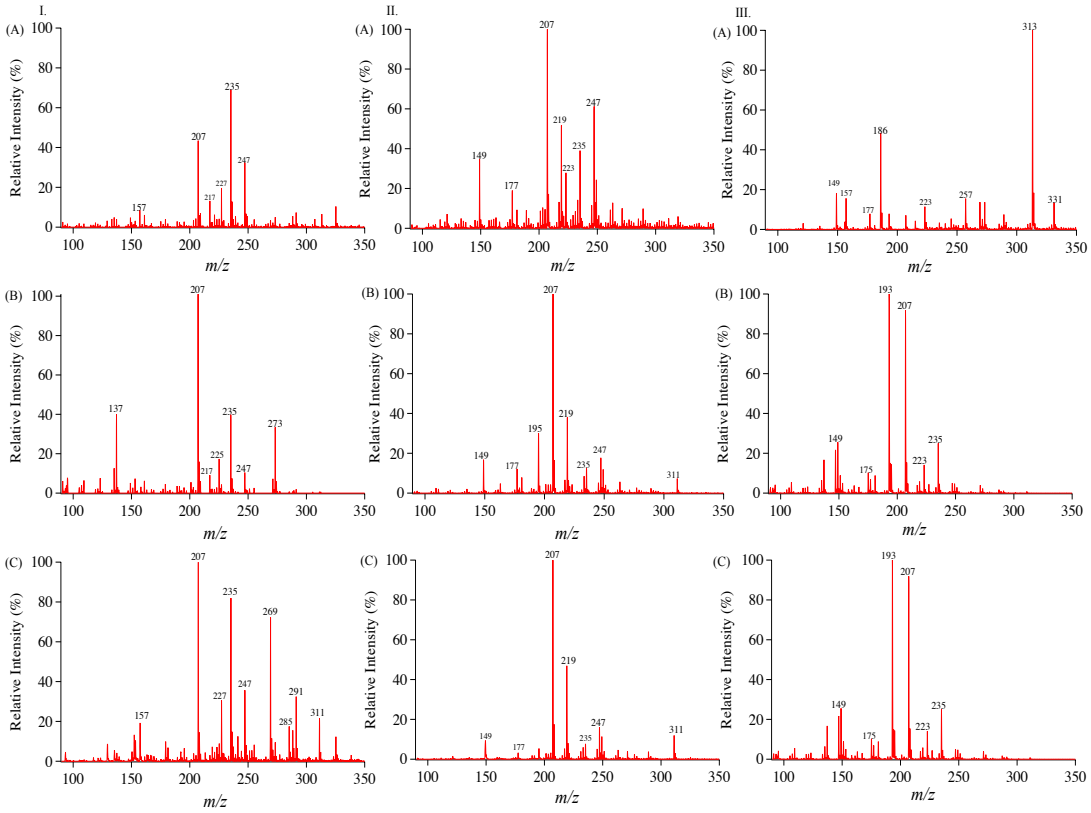


Figure 6.5. Mass spectra of the three men's authentic fragrances: I.) ´The one gentleman´by Dolce & Gabbana; II.) ´Encre Noire´by Lalique and III.)´Midnight in Paris´ by Van Cleef & Arpels obtained by electrospray ionization (A) and paper ESTASI-MS with the substrate of lint-free paper (B) or perfume testing strip (c) from the local shop. The spectra were obtained in a positive ion mode.

The most abundant peaks observed from the different perfumes by paper ESTASI-MS with lint-free paper as substrate are listed in table 6.2. The peak lists of the women's fragrances are not very different from those of the men's. Two peaks with *m/z* 137, 157 correspond to the protonated species of limonene and citronellol that can be found in the ingredients list for all the perfumes. The peak list obtained for all the perfume fragrances looks similar between each other suggesting that some same compounds are used for perfume preparation for women's and men's fragrances. However, in all commercial fragrances there are some specific peaks, which show the difference between the perfumes. The peaks with *m/z* 137, 207 and 235 are presented in several fragrances. The ion with *m/z* 151 was observed only in "Kenzo", *m/z* 263 in "Lalique", *m/z* 141 in "Hermes", *m/z* 273 in "D&G" and *m/z* 223 in "VCA". The perfumes are complex mixtures, containing not only chemicals giving odor but also compound for stabilization and dispersion, resulting in the different observed peaks.

Table 6.2. Peak list of the 11 most abundant peaks of the "Hermes", "Kenzo", "Dolce & Gabbana", "Lalique" and "Van Cleef & Arpels" perfume obtained from the lint-free paper by ESTASI-MS.

m/z	Hermes	Kenzo	D&G	Lalique	VCA
1	135	137	135	149	137
2	137	151	137	163	149
3	141	207	149	177	175
4	155	227	157	181	181
5	169	235	161	195	193
6	193	253	207	207	207
7	207	271	217	219	219
8	227	277	227	235	223
9	235	288	235	247	235
10	249	299	247	263	247
11	271	311	273	311	271

Non-volatile and odorless samples with *m/z* in the range of 100-300 can also be detected with the paper ESTASI-MS together with the perfume compounds. Indeed, these non-volatile and odorless samples (amino acid in our case) may be used as security labels for the perfume products to distinguish from the counterfeits. To demonstrate this concept, tyrosine was mixed with the "Givenchy" original fragrance and 5 μL of the solution was deposited on top of the lint free paper. After applying high voltage the main peak *m/z* 182 of a tyrosine and the main peaks of the fragrance were observed by MS (see Figure 6.6).

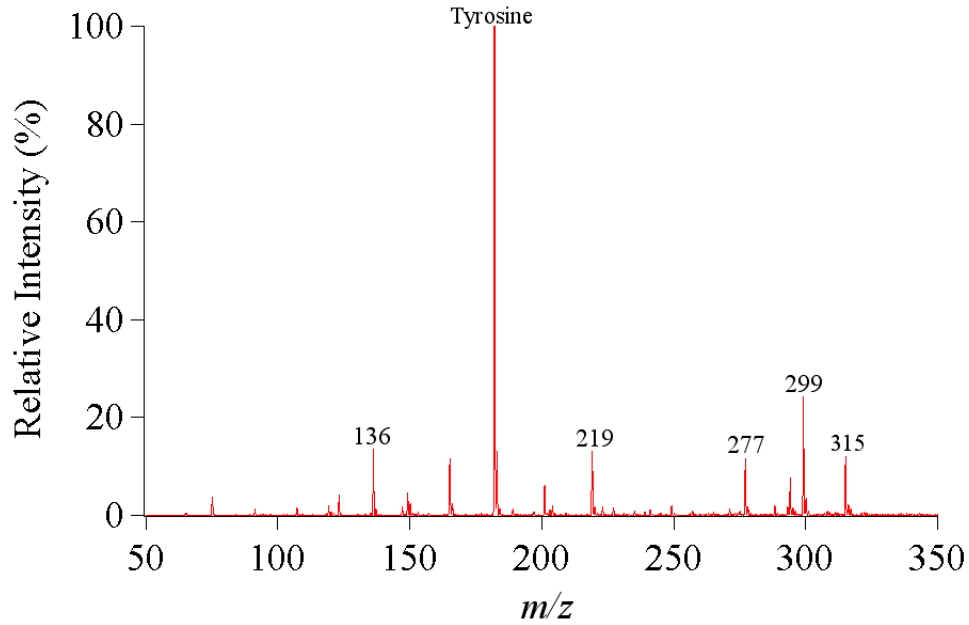


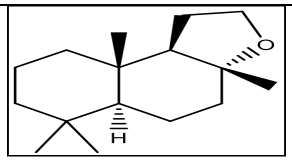
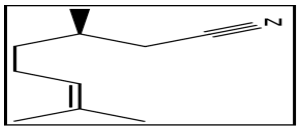
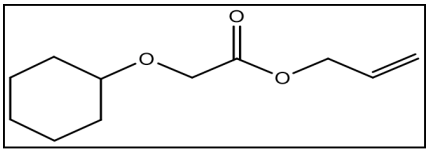
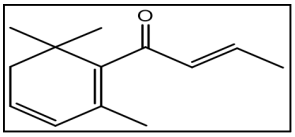
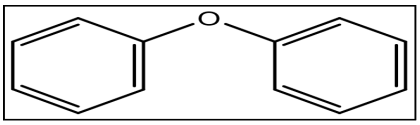
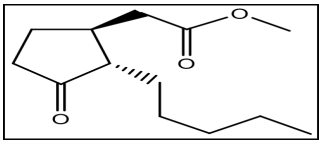
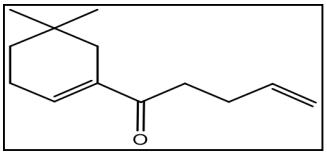
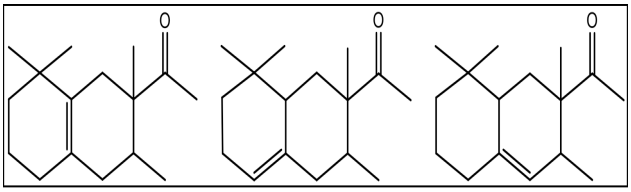
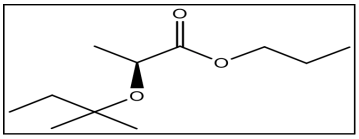
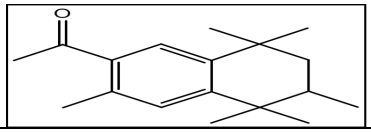
Figure 6.6. Mass spectra of Ange ou demon´ by Givenchy mixed with 10mM tyrosine solution. The MS data was obtained in a positive ion mode.

4. Conclusions

A strategy of rapid analysis of perfume fragrances by paper based electrostatic spray ionization mass spectrometry method was developed. A modified ion transfer capillary was used for simplifying the ion detection procedure. The spectra from the paper ESTASI-MS can be used as chemical fingerprints of perfumes. As an ambient ionization method for untreated samples, the paper ESTASI-MS is a high-throughput method for perfume analysis comparing to the ESI-MS by commercially equipped ESI source under direct-infusion mode by avoiding time-consuming cleaning steps. With these proof-of-concept results, we could expect this method to be applied in numerous research fields, such as quality control of perfume fragrances, aromatic and natural compounds, increasing the productivity of perfume and cosmetic industry.

Appendix 6.0

The structure and molecular weights of the compounds used in preparation of "model perfume" sample.

Constituents	Structure	Mol. Weight
AMBROX		236.34
CITRONELLYL NITRILE		151.25
CYCLOGALBANATE		198.26
DAMASCENONE		190.28
DIPHENYL ETHER		170
HEDIONE		226.32
NEOBUTENONE ALPHA		192.2
ISO E SUPER		234.38
SCLAREOLATE		202.15
TONALIDE PF		258.29

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Chapter VII.

Standard addition strip for quantitative electrostatic spray ionization mass spectrometry analysis: Determination of caffeine in drinks

Based on E. Tobolkina et al., Talanta, 2014, 130, 377-381.

1. Introduction

Many food and drinks with caffeine content are now commercially available. Consequently, there is a great interest for development of fast analytical tools to measure caffeine and other alkaloids, such as theobromine, theophylline and paraxanthine. These alkaloids have been found in more than 100 different plants and are widely used in the preparation of food, beverages and medicines with analgesic effect[1]. The most well known drinks containing caffeine are coffee and tea. Nowadays, these two beverages are the most consumed drinks all over the world[2]. Caffeine works as a natural drug to stimulate the central nervous and metabolic system[3]. It has positive influence, such as to prevent lung diseases and metabolic disorders[4]. However, its overconsumption can lead to caffeine overdose, in extreme cases to death[5]. The daily intake of caffeine is suggested to be less than 400 mg[6]. Therefore, it is important to quantify caffeine in various food and drink in order to control its consumption and even prevent caffeine intoxication.

Caffeine quantitative analyses have been performed with various analytical techniques, including high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection[7-9], capillary electrophoresis[10], gas chromatography (GC) coupled to mass spectrometry (MS)[11], HPLC-MS[12, 13], Fourier transform infra-red (FTIR) spectroscopy[14], high-performance thin-layer chromatography (HPTLC)[15] and *etc.* In addition to these widely used analytical methods, several ambient ionization MS techniques have been introduced for caffeine analysis, such as direct analysis in real time (DART) MS[16], probe electrospray ionization MS and low temperature plasma MS[17, 18]. Using ambient ionization MS can avoid time-consuming sample preparation procedures, therefore greatly shortening high throughput analyses.

Recently, a new contactless ambient ionization technique called electrostatic spray ionization (ESTASI) was developed in our laboratory, where a droplet of a liquid containing analytes on a plastic substrate is ionized by application of a pulsating high voltage (HV) for MS detection[19]. The main advantage of ESTASI over other electrospray techniques is that the samples require minimal or no preparation, meaning that ESTASI can provide fast and real-time measurements whilst generating accurate data. To date, ESTASI has been successfully used to analyze protein/peptide

dried on a plastic plate[19] or inside a porous matrix[20], to couple MS with capillary electrophoresis[19] and gel electrophoresis[20], as described in Chapter V, to quickly characterize perfume[21], as described in Chapter VI, and perform MS imaging of biomolecules on a substrate[22].

In this work, ESTASI-MS is applied for a rapid quantitative analysis of caffeine in beverages with only a few sample pretreatment procedures. The quantitative analysis method is based on plastic strips containing spots of standards at different concentrations, and is named as standard addition strip-ESTASI-MS. The quantification of caffeine in various samples has been performed both with the standard addition strip-ESTASI-MS method and compared with classical standard addition methods for MS or LC. Proof of concept results have been obtained, indicating that this strip strategy can be applied for fast and accurate food analysis and quality control.

2. Materials and methods

2.1 Chemicals & Materials

Caffeine (≥ 99%) and theobromine (≥ 99%) were obtained from Sigma-Aldrich (Steinheim, Germany). Methanol (99.9%, HPLC grade) was purchased from Applichem GmbH (Darmstadt, Germany). Acetic acid (100%) was obtained from Merck (Zug, Switzerland). All these reagents and materials were used as received without further purification. Black coffee, tea, and soft drinks (Coca-Cola™, Coca-Cola Zero™, Nestea™) were purchased from the local store. Deionized (DI) water (18.2 MΩ cm) was purified by an alpha Q Millipore system (Zug, Switzerland) and used for all experiments. Gelbond PAG film, 0.2 mm thickness, from Amersham Pharmacia Biotech AG (Uppsala, Sweden) was used as the insulating plate.

2.2 Sample Preparation

Soluble coffee (2 g) was diluted in 100 ml of boiled water. Black tea leaves (2 g) were poured with 100 ml of boiling water and filtered. Soft drinks (Coca-Cola™, Coca-Cola Zero™, Nestea Lemon™) were decarbonised using the sonication bath. All liquid samples were diluted 50 times with an acidic solution (50% methanol, 49% water and 1% acetic acid). In standard addition methods, pure caffeine of different concentrations was added into the diluted samples till a final concentration between 5 and 50 μg/ml. In all MS experiments, a fixed amount (25 μl) of an aqueous solution containing 200 μg/ml of theobromine was added into 975 μl of each diluted sample as an internal standard, giving a final concentration of 5 μg/ml.

2.3 High performance liquid chromatography quantitation of caffeine

HPLC analyses of caffeine were performed on a Waters 1525 (Milford, MA, USA) apparatus equipped with a binary pump, a Rheodyne injector with a 5 μL injection loop and a dual wave length UV detector 2487. The compounds were separated on a 4.6 mm i.d. × 250 mm, 5 μm particle reversed-phase Nucleodur C_{18} gravity column (Macherey-Nagel, AG, Switzerland). Methanol/water 50/50 (v/v) was used as ~~a~~ mobile phase. Isocratic elutions were performed at a flow rate of 0.8 ml/min. The column was operated at room temperature and the adsorption wavelength was set at

280 nm. The analysis time was 7 minutes. 20 μl of black tea or black coffee infusion previously diluted 50 times in the acidic solution, with or without pure caffeine additions (from 5 to 50 μg/ml) were injected. For each sample, experiments were repeated three times. Caffeine in the samples was identified by comparing its retention time with that of pure caffeine standard.

2.4 ESTASI-MS

A linear ion trap mass spectrometer of Thermo LTQ Velos (Reinach, Switzerland) with an ion transfer capillary modified into an "L" shape[21] was used for ESTASI-MS. Electrospray voltage of the LTQ Velos was set as 0 V. An enhanced ion trap scanning rate (10,000 *m/z* units/s) was used for the MS analysis. During the experiments, the commercial ion source of the instrument was replaced by an ESTASI ion source. 5 μl of sample in the acidic solution (50% methanol, 49% water and 1% acetic acid) was loaded on top of a polymer insulating plate by Eppendorf pipette. An electrode was placed behind the droplet and under the insulating plate. The distance between the MS inlet and the droplet was around 2 mm. By applying HV (9 kV) to the electrode, the droplet became polarised and as soon as the charge was large enough at the tip of the droplet, a spray of charged microdroplets occurred. When grounding the electrode, a spray of counter charges took place to re-establish the electroneutrality of the droplet. By applying a pulsating square wave HV (0 to 9 kV) at a given frequency, alternating spray of cations and anions took place. The square wave HV was amplified from a function generator by an amplifier (10HVA24-P1, HVP High Voltage Products GmbH, Martinsried/Planegg, Germany). A digital oscilloscope was used to monitor current and HV pulse. All mass spectra were recorded in positive ion mode. Data analysis was performed by Xcalibur Qual Browser (ThermoFisher Scientific, Reinach, Switzerland). More detailed information on ESTASI can be found elsewhere[19].

2.5 Standardization on ESTASI-MS

To determine the concentration of caffeine in unknown sample, theobromine (5 μg/ml) was mixed with pure caffeine of various concentrations (5-50 μg/ml) in an acidic solution containing 50% methanol, 49% water and 1% acetic acid. The

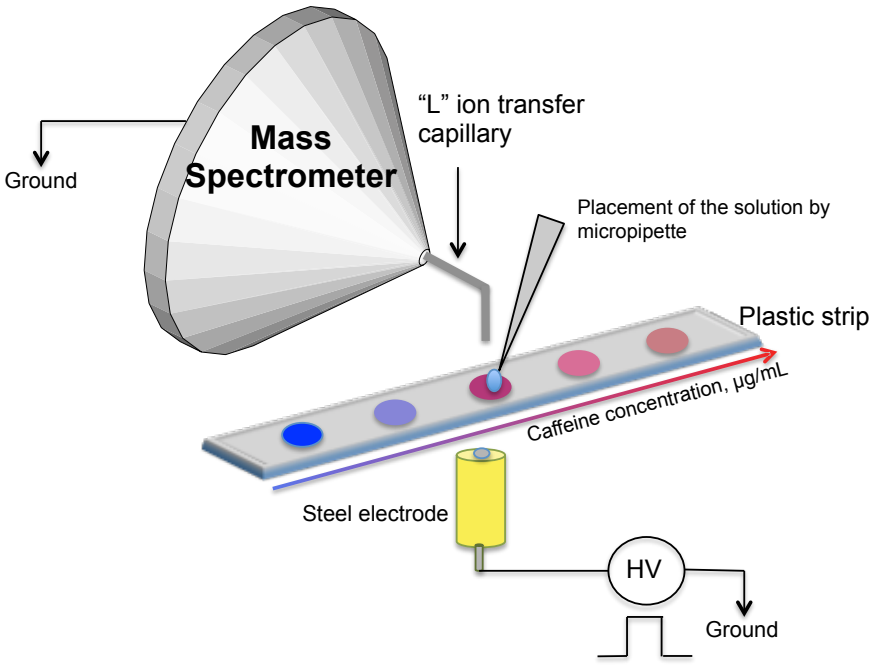
mixtures were analysed by ESTASI-MS, and the ratios of the peak intensities of caffeine and theobromine were plotted as a function of the caffeine concentration to demonstrate the quantitative analysis performance of ESTASI-MS.

2.6 Standard addition calibration by ESTASI-MS

The method of standard addition by ESTASI-MS was performed by adding small amounts of pure caffeine into the diluted samples in the acidic solution that contained also the internal standard of theobromine for ESTASI-MS analyses. Peak intensities of caffeine and theobromine from mass spectra were used for calculation. The experiments for each sample were repeated several times for accurate calibration and to obtain the standard deviation (SD). The calibration curves and figures were plotted using IGOR Pro (Version 6.00 for Macintosh, WaveMetrics, Lake Oswego, OR, USA).

2.7 Standard addition strip-ESTASI-MS

Arrays of wells (1 mm diameter, 2 mm depth) were drilled on a plastic strip using a driller. 5 μl of pure caffeine of known concentration (5-50 μg/ml) was deposited in the wells and left to dry. The strip containing the dried spots of pure caffeine was placed under the MS inlet, and 5 μl of a diluted sample mixed with theobromine in the acidic solution was loaded directly on top of each dried spot just before ESTASI-MS analysis. The electrode and ion transfer capillary were set in such a way to be in line with the center of the drilled hole to achieve good reproducibility. The obtained signal intensities of caffeine and theobromine were used to calculate the caffeine amount in the sample.



Scheme 7. 1. Schematic representation of the electrostatic spray ionization from a plastic strip containing the dried droplets of caffeine spots, HV: high voltage.

3. Results and discussion

3.1 Quantitative analysis from droplets of standard solution by ESTASI-MS

The limit of detection (LOD) for caffeine by ESTASI-MS was found as 51 nM (see Figure 7.1).

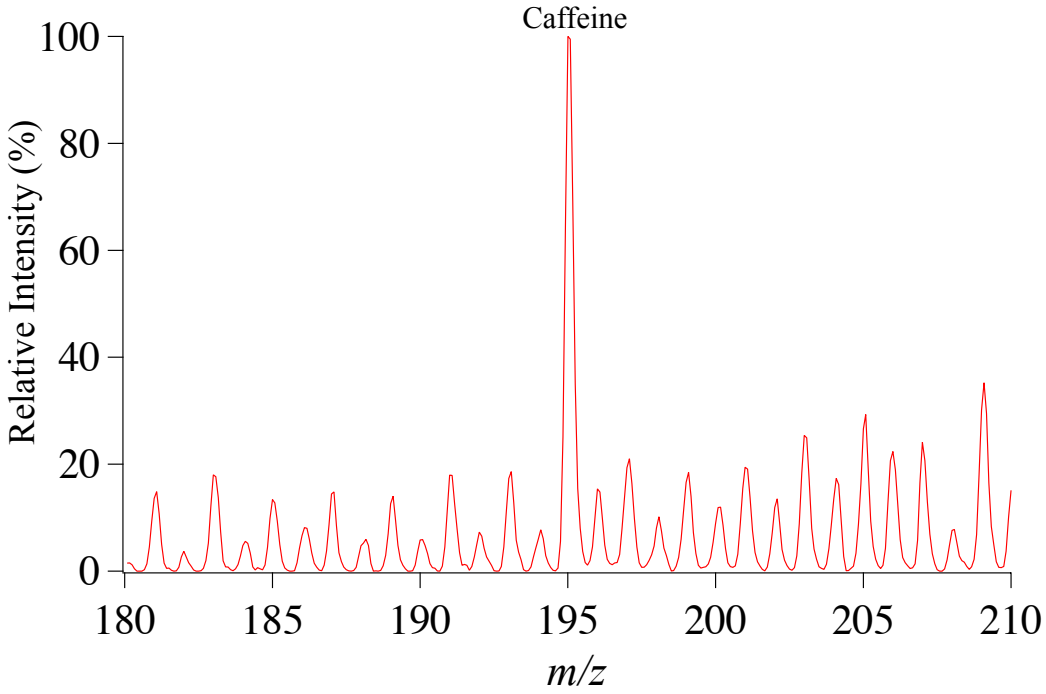


Figure 7.1. Mass spectrum of caffeine with the concentration of 51 nM obtained under positive MS mode. The ions were generated by ESTASI-MS when a pulsed positive high potential (9.0 kV) was applied to the electrode.

To demonstrate the quantitative analysis performance, a series of standard solution was prepared in 50% methanol, 49% water and 1% acetic acid containing pure caffeine at different concentrations (5-50 μg/ml) and a constant amount of internal standard (5 μg/ml) of theobromine. Theobromine was selected as the internal standard since it has a structure similar to that of caffeine. 5 μl of each solution containing a different concentration of caffeine was deposited on an insulating plate for ESTASI-MS analysis. As shown in Figure 7.2, both caffeine and theobromine were detected as single protonated ($[M+H]^+$) ions in a positive ion mode. The peak at *m/z* 195.1 corresponds to caffeine and the peak at *m/z* 181.2 corresponding to theobromine (see Figure 7.2.a). The intensities ratios were calculated and plotted against caffeine concentrations to get a calibration curve (see Figure 7.2.b). The good linearity shows

that ESTASI-MS is a technique suitable for quantitative analysis, and that the theobromine is a suitable internal standard for caffeine quantification by MS.

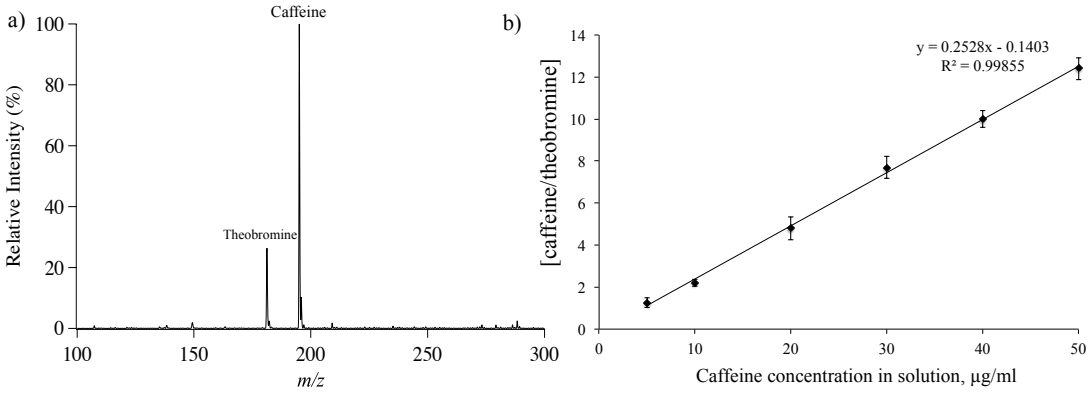


Figure 7.2. a) Positive mode ESTASI-MS signal of 10 μg/ml caffeine solution mixed with 5 μg/ml of theobromine in 50% methanol, 49% water and 1% acetic acid obtained under a frequency of the square wave HV (0 to 9 kV) as 40 Hz. **b)** The mass spectral peak intensity ratio between caffeine and theobromine as a function of the caffeine concentration. Error bar shows the standard deviation calculated from three experiments.

3.2 Standard addition calibration of caffeine in various drinks by ESTASI-MS

The method of standard addition was used to calculate caffeine concentrations in different drinks by ESTASI-MS. All the drinks were diluted 50 times in the acidic solution and then mixed with different concentrations of pure caffeine and the internal standard of theobromine at fixed concentration. The concentrations of added pure caffeine were selected in a way that they were close to the concentration of caffeine in the test sample for accurate quantifications. 5 μl of each mixture was deposited on a plastic strip (Gelbond PAG film) and analyzed directly by ESTASI-MS. The changes in intensities between caffeine and theobromine were observed and used for calculating the calibration curves. The calibration curve for a coffee sample is shown on Figure 7.3. From this curve, the concentration of caffeine in this coffee was calculated as 272 μg/ml.

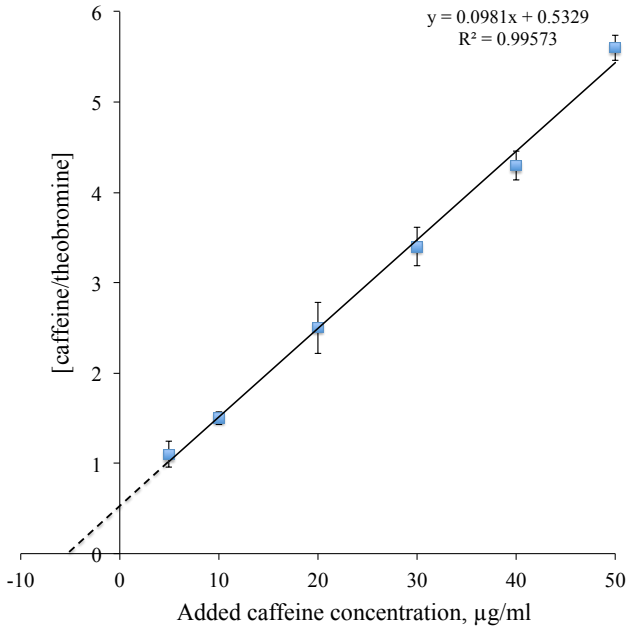


Figure 7.3. Plot of caffeine/theobromine single protonated peak intensity ratio in the presence of coffee obtained by the method of standard addition using ESTASI-MS. Error bar shows the standard deviation calculated from three experiments.

Soft drinks, such as Coca-Cola™, Coca-Cola Zero™, and Nestea™, as well as tea extracts were also analyzed using ESTASI-MS by the standard addition method. The data obtained were plotted to calculate the caffeine concentrations in different beverages (see Figure 7.3.1), and summarized in Table 7.1.

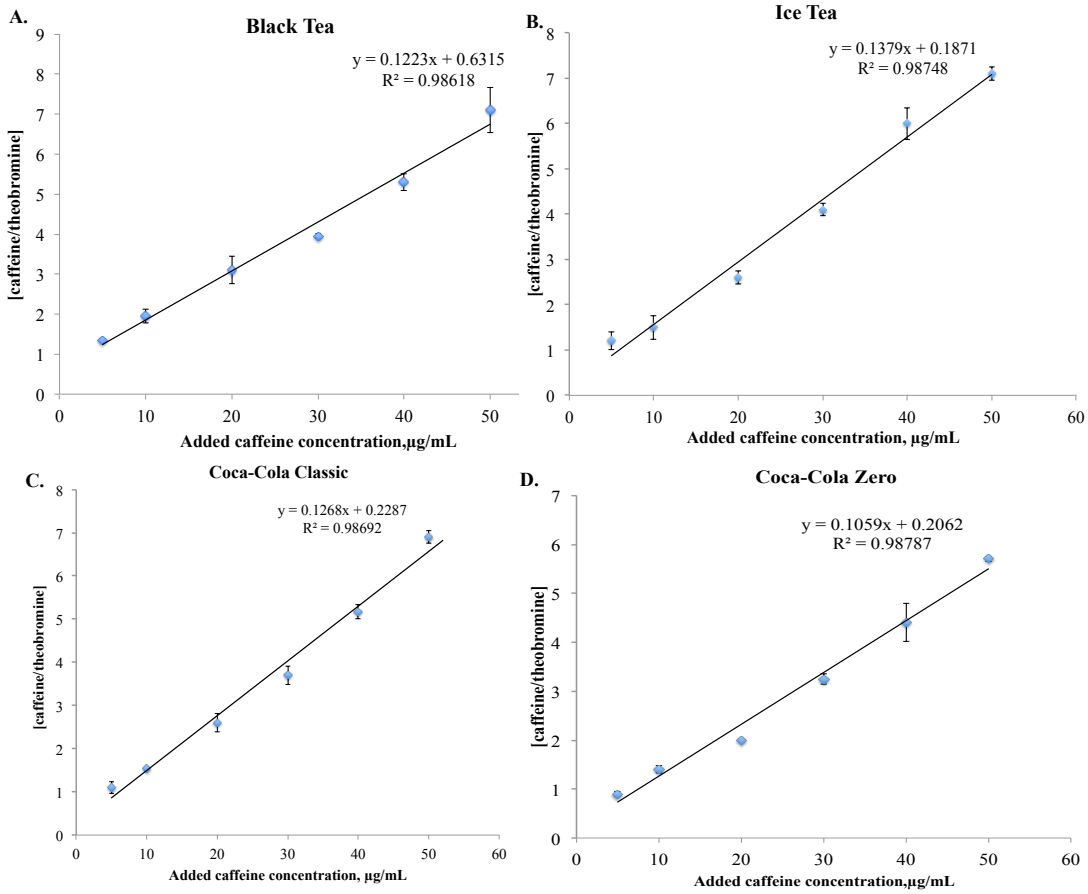


Figure 7.3.1. The plot of caffeine/theobromine signal intensity ratio in the presence **A.** Black tea infusion, **B.** Ice tea, **C.** Coca-Cola Classic and **D.** Coca-Cola Zero as a function of the added caffeine concentrations obtained by ESTASI-MS. Error bar shows the standard deviation calculated from three experiments.

Table 7.1. Caffeine concentrations in the samples of different beverages calculated by methods of standard addition and standard addition strip using ESTASI-MS.

Sample	C_{MSD}, µg/ml*	C_{Strip}, µg/ml	C, µg/ml
Black coffee infusion	271.61 ± 0.32	295.65 ± 0.24	281.81± 0.38[A]
Black tea infusion	258.18 ± 0.52	259.98 ± 0.01	245.21±0.62[A]
Coca-Cola Zero	97.36 ± 0.17	98.17 ± 0.21	96[B]
Coca-Cola Classic	90.18 ± 0.70	95.50 ± 0.11	95[B]
Ice tea	67.84 ± 0.41	70.88 ± 0.13	68-72[B]

* calculated as undiluted drink
A: concentration of caffeine obtained by classic HPLC method
B: reported concentration from the manufacturer
C_{MSD}: concentration calculated by method of standard addition
C_{strip}: concentration calculated by standard addition strip-ESTASI-MS

For soft drinks, the caffeine concentrations obtained using the ESTASI-MS were

compared with the values reported by the manufacturer. For the black coffee and tea infusions, the caffeine concentration obtained by ESTASI-MS was compared with results from classic HPLC method, since the caffeine concentrations could vary from sample to sample depending on the preparation procedures (see Figure 7.4.1- 7.4.2). As shown in Table 7.1, the caffeine concentrations obtained by ESTASI-MS are in good agreement with the reported values and the ones obtained by HPLC measurements, indicating that the ESTASI-MS is an efficient approach for caffeine quantitative analysis. With the experiments repeated three times for all examined samples, the standard deviations were calculated and presented in Table 7.1. Between the experiments, the MS ion transfer capillary was washed in order to prevent carry over of caffeine that can adsorb on the inner walls of the capillary.

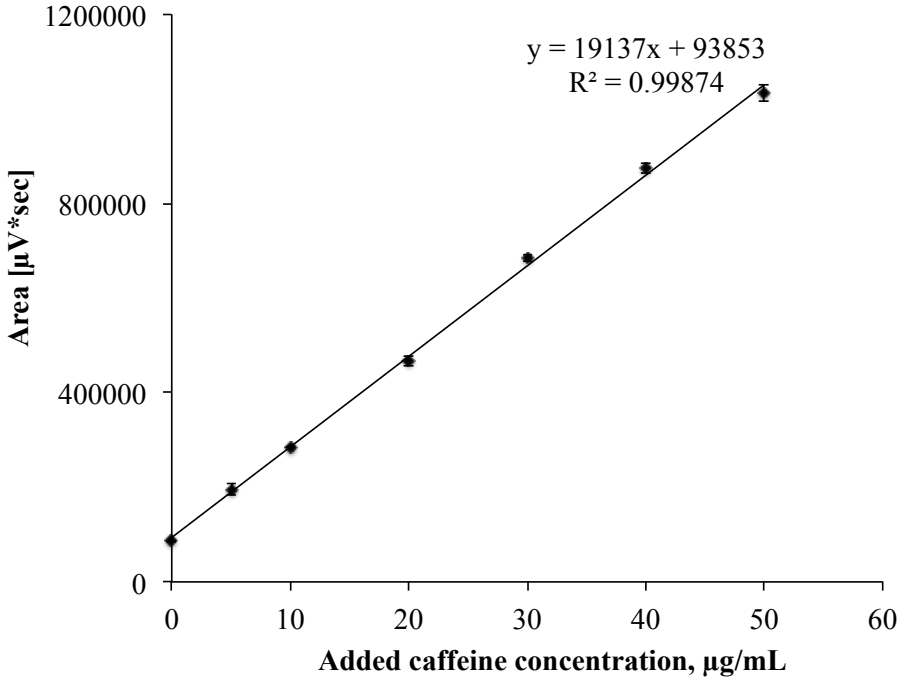


Figure 7.4.1 The plot of average area of a caffeine peak in the presence of black tea extract ("Lipton") as a function of the added caffeine concentrations obtained by HPLC. Error bar shows the standard deviation calculated from three experiments.

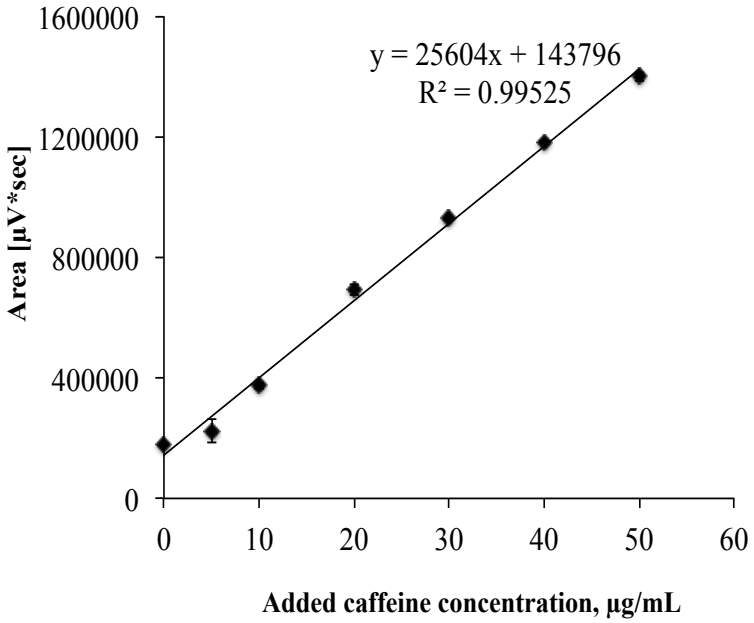


Figure 7.4.2 The plot of average area of a caffeine peak in the presence of coffee ("Nescafe Gold") as a function of the added caffeine concentrations obtained by HPLC. Error bar shows the standard deviation calculated from three experiments.

The main advantage of using standard addition method is to avoid matrix effect from complex samples. In a real drink sample, many molecules may be ionized that could influence the ionization of caffeine and theobromine. This influence is obvious when the standard addition calibration was performed with soft drinks, coffee or tea. The suppression in food samples was previously reported[23, 24].

3.3 Method of standard addition using strip-ESTASI-MS

We present here a method of calibration based on the preparation of a plastic plate containing a series of dry spots of the analyte of interest to be examined by ESTASI-MS. A plastic strip of appropriate length with wells of 1 mm in diameter was employed. 5 μl droplets containing caffeine at different concentrations in the range of 5-50 μg/ml in water were deposited on the wells and dried under ambient conditions. The wells were used to help localization and controlled sample deposition. The plastic strip can be directly used for caffeine quantification or stored for future tests.

Using the strip as an insulating plate for ESTASI, quantitative analysis of caffeine in drinks can be directly performed without a preparation step of mixing the sample with

the dried pure caffeine, as illustrate in Scheme 7.1. The mixture (5 μl) containing constant amount of theobromine and a test sample in acidic solution was loaded on each dried spot. The dried caffeine on the spot was dissolved, immediately extracted and ionized together with the caffeine in the test sample and theobromine for ESTASI-MS analysis (see Figure 7.6).

It was also verified that the previously dried pure caffeine was fully extracted from the surface of a strip during ESTASI-MS analyses (see Figure 7.5). For that droplets (5 μl each) of caffeine/theobromine mixture in the acetic solution containing different concentrations of pure caffeine (5, 10, 20, 30, 40, 50 μg/ml) were deposited on a blank strip and a strip with previously dried caffeine spots. The mass spectral intensity ratios between caffeine and theobromine were calculated and plotted as a function of the caffeine concentration as shown on Figure 7.2.b. Curve A with a gradient of 0.2528 was obtained from the blank strip; while curve B with a gradient of 0.5003, almost double of curve A, was obtained from the strip with previously dried spots containing 25, 50, 100, 150, 200, 250 ng of caffeine, respectively. Such a result indicates that almost all previously dried caffeine on the spots was quickly dissolved into the acidic droplet for ESTASI-MS analyses.

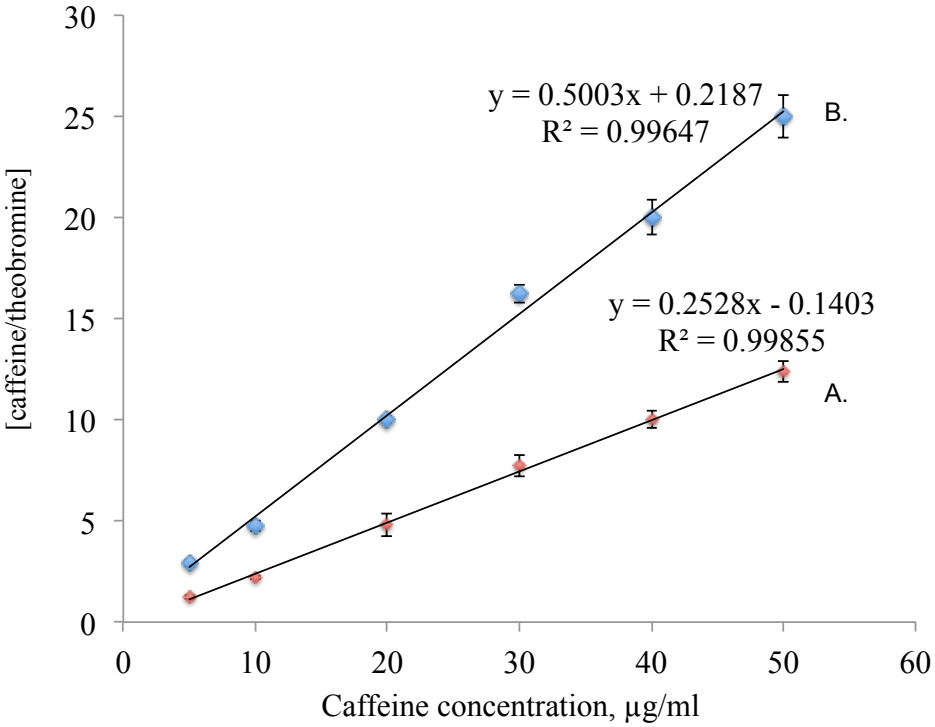


Figure 7.5. The mass spectral peak intensity ratio between caffeine and theobromine as a function of the caffeine concentration obtained from droplets of acidic solutions containing varied concentrations of caffeine and a fixed concentration of theobromine on a blank strip (A) and on a strip containing previously dried caffeine spots (B). In the latter case, the amounts of caffeine on the dry spots were kept same as the ones within the acidic solution droplet. Error bar shows the standard deviation calculated from three experiments.

The spots were analyzed in the order of increasing the concentration of the previously dried caffeine. All mass spectra were recorded in positive ion mode. The intensities of caffeine and theobromine were detected by MS and further used to plot the calibration curve (see Figure 7.6-7.6.1). When the test sample was black coffee infusion, analytical response of caffeine showed a good linear correlation (y=0.0191x + 0.5647) and a determination coefficient R^2 of 0.995. With this calibration curve, the caffeine amount was found by strip-ESTASI-MS as 1478 ng, corresponding to a concentration of caffeine in coffee as 295 μg/ml.

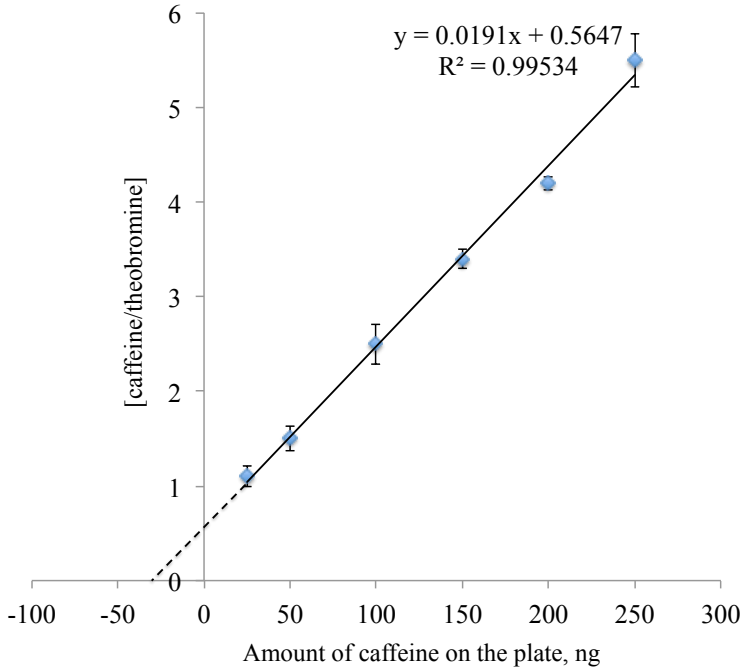


Figure 7.6. Standard plot of caffeine/theobromine mass spectral signal intensity ratio in the presence of coffee performed using the strip-ESTASI-MS. The standard deviation was calculated from three experiments and shown as the error bar.

As it can be observed, the calibration curve for the strip-ESTASI-MS standard addition is quite similar to the one for normal standard addition shown as Figure 7.2, meaning that the method of strip-ESTASI-MS could be used as a convenient, rapid and efficient method for calculating caffeine concentrations in different beverages.

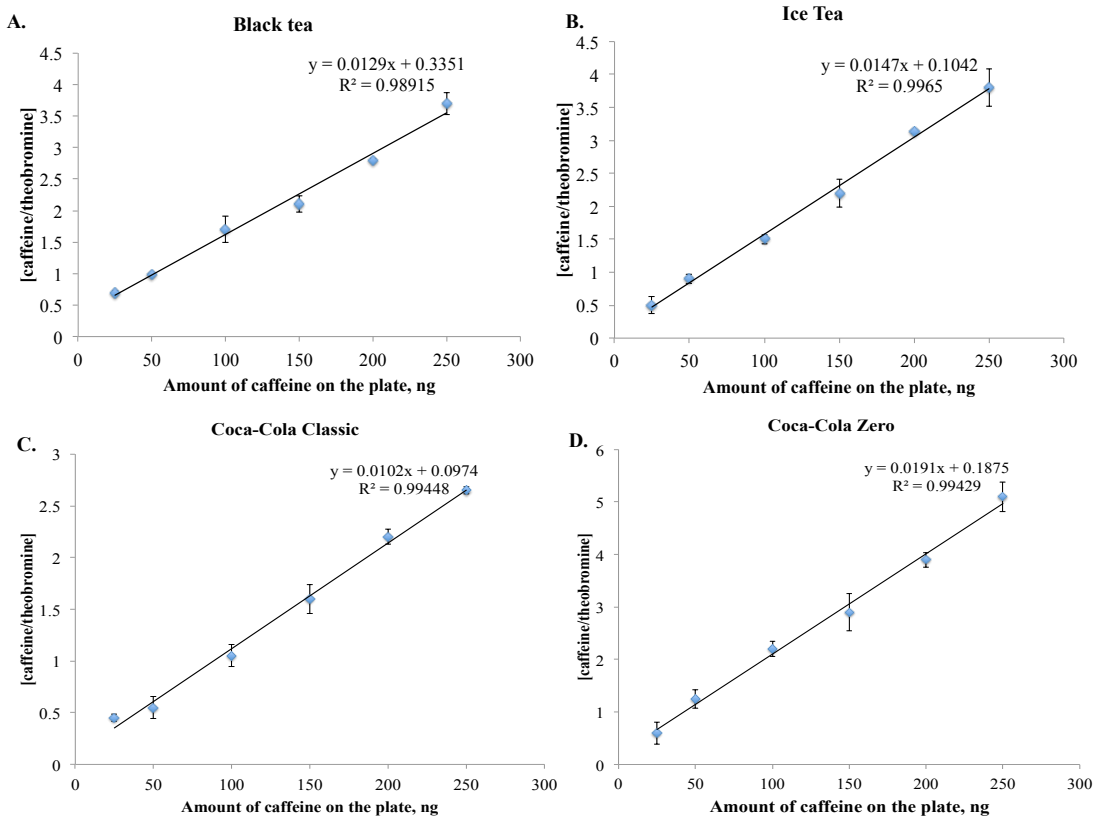


Figure 7.6.1 The plotting of caffeine/theobromine signal intensity ratio in the presence of **A.** Black tea infusion; **B.** Ice tea; **C.** Coca-Cola Classic; **D.** Coca-Cola Zero as a function of amount of loaded pure caffeine on the strip by the strip-ESTASI-MS standard addition method. The standard deviation was calculated from three experiments and shown as the error bar.

The caffeine concentrations in different beverages obtained by the strip-ESTASI-MS are also summarized in Table 7.1. The values are in a good agreement with the reported values and those from the classical standard addition method without strip. The plotted curves of caffeine/theobromine mass spectral intensity ratio in the presence of different matrices (soft drinks and tea infusion) are presented in 7.6.1. It should be noted that during the analysis of different beverages, not only caffeine and theobromine were detected by MS, but also other compounds present in the sample. For instance the mass spectra recorded in a positive ion mode by ESTASI-MS of Nestea Lemon is presented in Figure 7.7.

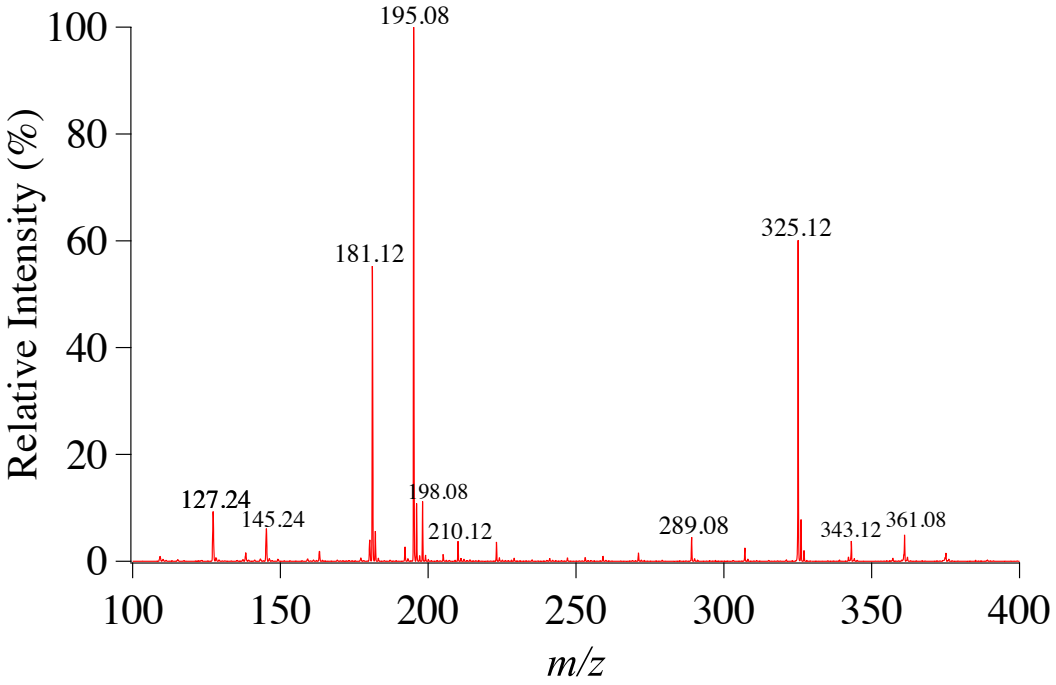


Figure 7.7. Mass spectra of Nestea Lemon obtained by strip-ESTASI-MS. The caffeine amount on the strip was 50 ng. The MS data was obtained in a positive ion mode.

3.4 Quantification of caffeine in saliva by strip-ESTASI-MS

The strip-ESTASI-MS standard addition method was applied for caffeine quantification in saliva. The saliva was collected at different times from a healthy volunteer after drinking a cup of coffee. The collected samples of saliva were spiked with constant amount (5 μg) of internal standard. The concentration of caffeine in each sample was analyzed using the standard addition strip-ESTASI-MS. The change in caffeine concentration with time is presented on Figure 7.8.

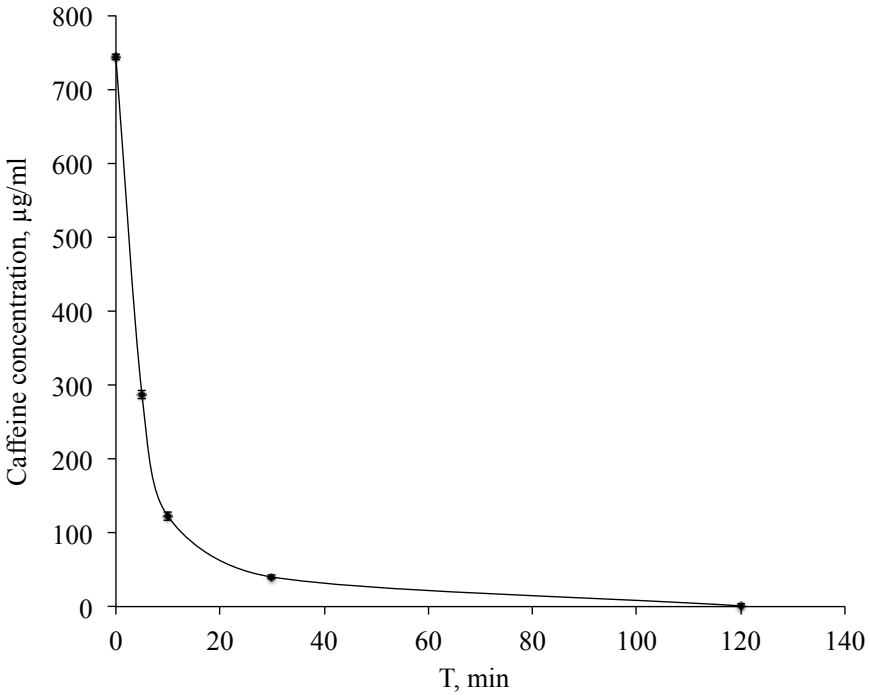


Figure 7.8. Caffeine concentration in saliva 0, 5, 10, 30, 120 min after drinking coffee determined by strip-ESTASI-MS standard addition method. The standard deviation was calculated from three experiments and shown as the error bar.

As it is seen from Figure 4, the caffeine concentration decreased with time as expected. The concentration of caffeine in saliva directly after drinking the black coffee infusion from the local cafeteria was 743 μg/ml (see Figure 7.9). In 5 min the concentration reduced to 287 μg/ml, in 10 min to 122 μg/ml and in 30 min to 30 μg/ml. Even after 2 hours the caffeine was still presented in the saliva and detected by ESTASI-MS with an amount of 1 μg/ml, which is much higher than the LOD of ESTASI-MS to caffeine of 10 ng/ml as explained before. We would expect to apply this method for the fast analysis of caffeine not only in saliva, but also in blood, urine or samples of interests in the view of forensic science.

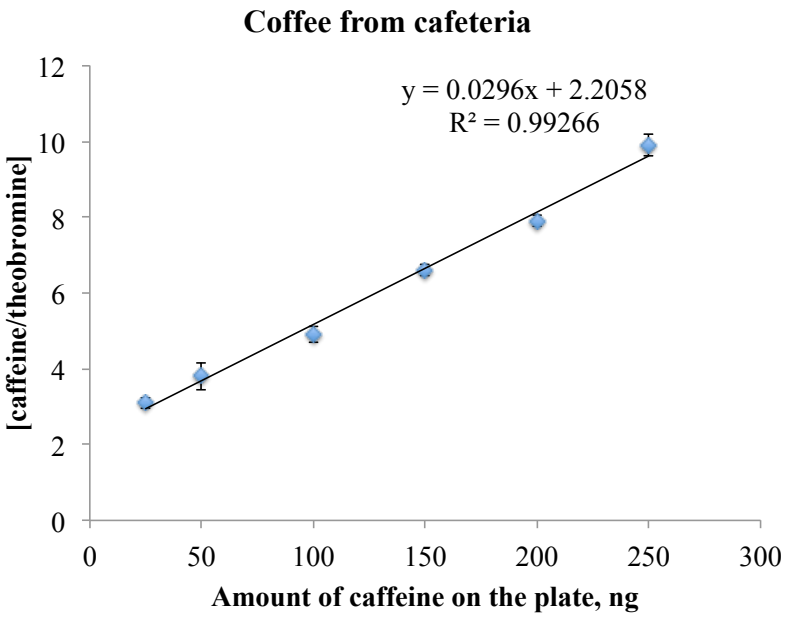


Figure 7.9. The plotting of caffeine/theobromine signal intensity ratio in the presence of saliva directly after drinking a cup of coffee from the local cafeteria as a function of amount of caffeine on the plate by the strip-ESTASI-MS standard addition method. The standard deviation was calculated from three experiments and shown as the error bar.

4. Conclusions

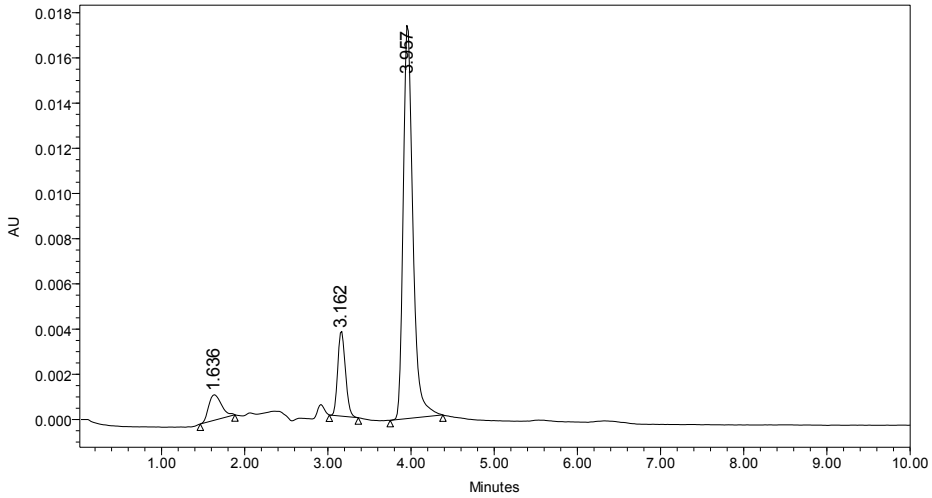
ESTASI-MS has been used as a quantitative technique for caffeine analysis. This new ambient ionization method can be used for fast and high-throughput quantification of different compounds in samples of interest. The method of standard addition by ESTASI-MS based on a strip containing dried spots of caffeine had been used for caffeine analysis in the different beverages, such as soft drinks (Coca-Cola™, Coca-Cola Zero™, Ice tea), coffee and tea extract. The calculated values were compared with the reported ones, and are in a good agreement. This new method can be applied in numerous research fields, such as quality control of soft drinks, coffee, tea, food, tobacco, and alcohol industry.

Appendix 7.0

SAMPLE INFORMATION

Sample Name:	solution1Unk.	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	29.04.2014 13:45:19
Vial:	1	Acq. Method:	IPS
Injection #:	1	Processed By:	System
Injection Volume:	5.00 ul	Date Processed:	29.04.2014 13:56:20
Run Time:	10.00 Minutes	Channel Name:	2487Channel 2
Sampling Rate:	1.00 per sec	Channel Desc.:	
		Sample Set Name:	

Sample Values
Used in Calculations:



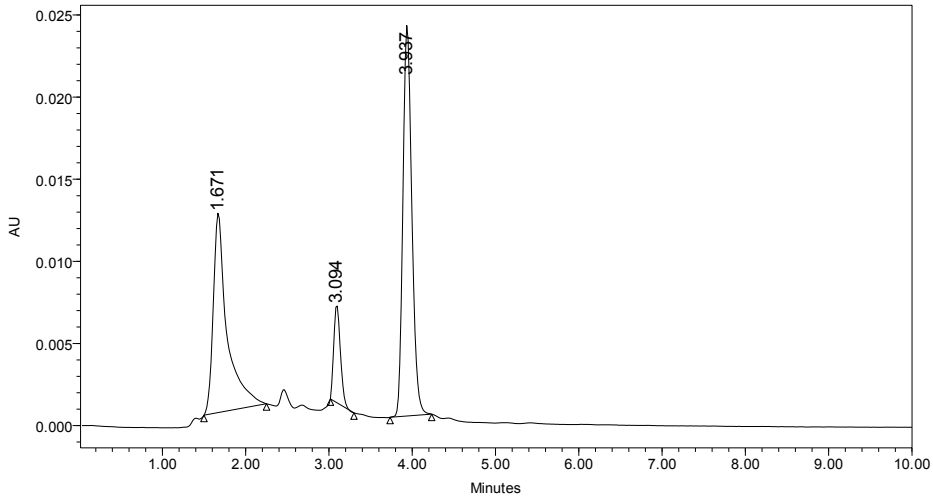
	RT (min)	Peak Type	Area (µV*sec)	% Area	Height (µV)	% Height	Integration Type	Points Across Peak	Start Time (min)	End Time (min)	Baseline Start (min)
1	1.636	Unknown	12022	6.79	1129	5.04	bb	25	1.467	1.883	1.467
2	3.162	Unknown	24168	13.65	3759	16.80	bb	21	3.017	3.367	3.017
3	3.957	Unknown	140819	79.55	17493	78.16	bb	37	3.750	4.383	3.750

Figure A-7.1 Chromatogram obtained for the determination of caffeine in a black tea sample using 50/50 MeOH/water mobile phase. The peak at 3.957 min corresponds to caffeine.

SAMPLE INFORMATION

Sample Name:	coffee 1.1 Unk.	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	30.04.2014 10:16:01
Vial:	1	Acq. Method:	IPS
Injection #:	9	Processed By:	System
Injection Volume:	5.00 ul	Date Processed:	30.04.2014 10:36:59
Run Time:	10.00 Minutes	Channel Name:	2487Channel 2
Sampling Rate:	1.00 per sec	Channel Desc.:	
		Sample Set Name:	

Sample Values
Used in Calculations:



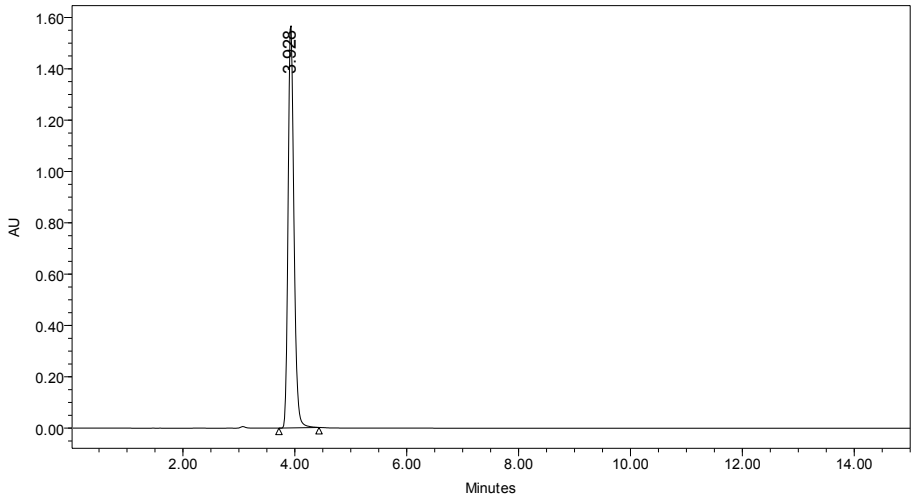
	RT (min)	Peak Type	Area (µV*sec)	% Area	Height (µV)	% Height	Integration Type	Points Across Peak	Start Time (min)	End Time (min)	Baseline Start (min)
1	1.671	Unknown	140478	40.00	12132	29.02	bb	45	1.500	2.250	1.500
2	3.094	Unknown	32952	9.38	5902	14.12	bb	16	3.017	3.300	3.017
3	3.937	Unknown	177730	50.61	23767	56.86	bb	30	3.733	4.233	3.733

Figure A-7.2. Chromatogram obtained for the determination of caffeine in a black coffee sample using 50/50 MeOH/water mobile phase. The peak at 3.937 min corresponds to caffeine.

SAMPLE INFORMATION

Sample Name:	caffeine 5050Unk.	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	29.04.2014 10:37:33
Vial:	1	Acq. Method:	IPS
Injection #:	1	Processed By:	System
Injection Volume:	5.00 ul	Date Processed:	29.04.2014 13:59:09
Run Time:	15.00 Minutes	Channel Name:	2487Channel 2
Sampling Rate:	1.00 per sec	Channel Desc.:	
		Sample Set Name:	

Sample Values
Used in Calculations:



	RT (min)	Peak Type	Area (µV*sec)	% Area	Height (µV)	% Height	Integration Type	Points Across Peak	Start Time (min)	End Time (min)
1	3.928	Unknown	11406046	100.00	1571351	100.00	bb	43	3.717	4.433

Figure A-7.3. HPLC chromatogram of 0.5 mg/ml pure caffeine separated using 50/50 MeOH/water mobile phase.

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Chapter VIII.

Direct analysis of desalted samples from RP-C_{18} ZipTip® by electrostatic spray ionization mass spectrometry

Based on E. Tobolkina et al., to be submitted.

1. Introduction

Mass spectrometry (MS) is an analytical method, which plays a principle role in protein characterization and identification. Since the proteomic sample is normally in a highly complex matrix and can contain up to several hundreds of different proteins, separation techniques are required before the MS analysis[1]. There is a big advantage of using separation methods prior to enzymatic or chemical digestions, since digestion is then made on a single type of proteins followed with MS characterization, where normally a higher sequence coverage of proteins as well as information on post-translational modifications (PTMs) can be obtained[2]. The most popular methods used for protein separation include gel electrophoresis, capillary electrophoresis and liquid chromatography[3].

The characterization of digested proteins relies mainly on two kinds of biological mass spectrometers, that are matrix-assisted laser desorption/ionization (MALDI)-MS and electrospray ionization (ESI)-MS. Since the late 1980s, ESI-MS became one of the most powerful, sensitive and reliable tools used to analyze different biomolecules[4], that can be easily coupled with liquid separation systems and realized in a highly automated analysis flow. However, one of the drawbacks that limit its application is the poor tolerance to salts leading to extensive sample preparation and low sensitivity[5].

Since high concentrations of salts in most biological fluids and detergents such as urea or sodium dodecyl sulfate (SDS) that can be introduced during sample preparation are incompatible with MS analysis, the proteins/peptides mixtures must be desalted prior to MS experiments. Several on-line and off-line strategies have already been utilized for sample desalting[6] prior ESI-MS analysis, such as using a trap column or filter before the analytical column in LC-MS, commercially available tools with the presence of chromatographic media in micropipette ZipTip® (Millipore)[7], UltraMicrospin® columns (Nest Group)[8] and Sep-Pak® cartridges (Waters)[9], and techniques such as dialysis[10], microchannel laminar flow devices[11] and size-exclusion chromatography (SEC)[12]. Among these methods, the most commonly used off-line technique to desalt and concentrate proteins are solid phase extraction (SPE)[13] and reversed-phase high performance liquid chromatography (RP-HPLC)[14, 15] due to the experimental simplicity and low sample volume requirement. Despite the development of various strategies, sample cleaning prior to ESI-MS

analysis is often not simple and time-consuming, meaning that there is still an interest in optimizing the desalting and pre-concentration strategies in facile ways.

Recently, we have introduced an ambient ionization method based on ESI named ElectroStatic Spray Ionization (ESTASI)[16]. The main difference of ESTASI from ESI is that the high voltage (HV) is applied on an electrode isolated from the sample solution by a layer of insulator, while the MS inlet plays the role of a counter electrode. When a positive high voltage pulse is applied on the electrode, a spray of cations happens during charging the capacitor formed by the electrode, insulator and sample solution. The spray of anions takes place afterwards when the electrode is grounded. During the spray of cations or anions, the generated ions are directly detected by mass spectrometer. ESTASI is a very flexible and easy to use ionization technique, which can be employed for different chemical and biochemical applications. Several of them have already been discussed in the previous chapters, including analyses of protein/peptide inside polyacrylamide gel[17], molecules separated by capillary electrophoresis (CE)[16], perfume fragrances from different type of papers[18], as well as imaging of melanoma cells[19].

In the present chapter, ESTASI-MS was applied for the direct analysis of samples after desalting using commercially available RP- C_{18} ZipTip micropipettes. Herein, we demonstrate that ZipTip-ESTASI-MS provides a fast sample cleanup method, while minimizing the experimental time, hence providing a rapid chemical or biochemical analysis of samples containing little- or non-volatile salts. Protein/peptide samples, containing different amounts of salts were used for the testing. The results obtained before and after ZipTip cleaning by ESTASI-MS analysis were compared with the ones achieved by the ESI-MS or the traditional ESTASI-MS from microdroplets deposited on a plastic plate. The main advantage of using the proposed approach is that the volume required is low, no sample preparation is required and experimental time is as short as less than 2 min.

2. Materials and methods

2.1 Chemicals & Materials

Cytochrome c (horse heart, 95%), sodium chloride (99.5%) and trifluoroacetic acid (TFA) were obtained from Fluka (St. Gallen, Switzerland). Angiotensin I (Ang I, NH_2−DRVYIHPFHL−COOH, 98%) was obtained from Bachem (Bubendorf, Switzerland). Caffeine (≥ 99%) was obtained from Sigma-Aldrich (Steinheim, Germany). Ciprofloxacin (CIP), Enrofloxacin (ENR), Lomefloxacin (LOM), Fleroxacin (FLE) were purchased from TCI Deutschland GmbH (Eschborn, Germany), and Ofloxacin (OFL) from Sigma-Aldrich (Schnelldorf, Switzerland). Methanol (99.9% HPLC grade) was purchased from Applichem GmbH (Darmstadt, Germany). Acetic acid (HAc, 100%) was obtained from Merck (Zug, Switzerland). Deionized water (18.2 MΩ cm) was obtained from an ultra-pure water system (Milli-Q 185 Plus, Millipore) and used for all experiments. Reversed-phase C_{18} (RP-C_{18}) ZipTip® pipette tips were obtained from Merck KGaA (Darmstadt, Germany).

2.2 ESTASI-MS

ESTASI-MS experiments were performed on an ion trap mass spectrometry (Thermo LTQ Velos, ThermoFisher Scientific, Reinach, Switzerland). The spray voltage of the internal power source of the MS was always set as 0. An enhanced ion trap scanning rate (*m*/*z* 10,000 per second) was used. The sample solution was sprayed from the normal micropipette tip or from the RP-C_{18} ZipTip® pipette tips.

To spray from a micropipette tip, 5 μl of a solution containing the sample mixed with an ESI buffer (50% (v/v) methanol, 49% water and 1% acetic acid) was loaded previously to round disposable tip using the micropipette. A silver wire, used as an electrode was coiled around the tip to form several loops (see Figure 8.1). Afterwards, as shown in Figure 8.1, the tip was placed in a protective plastic cup to prevent possible sparks since the distance between the metallic part of MS and electrode is rather short ≈ 10 mm. The back part of the protective cup is covered with a paraffin film to keep the tip stable during the experiment. To induce ESTASI, a high voltage square wave pulse (from 0 V to 9 kV, frequency 5 Hz or 40 Hz, limiting current 0.1 mA) was generated with a high voltage amplifier 10HVA24-P1 (HVP High Voltage Products GmbH, Martinsried/Planegg, Germany) and a function

generator (TG 315, Aim-TTi instruments, Cambridgeshire, England) between the microelectrode and the MS inlet.

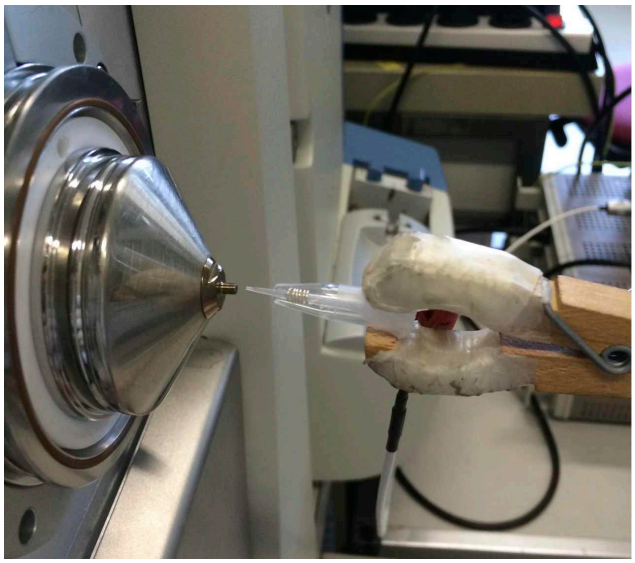


Figure 8.1. Solution is sprayed from the pipette tip by ESTASI to produce ions for MS analysis.

The sample of proteins and peptides containing different concentrations of salt (0.01M, 0.1M and 1M of $NaCl^-$) were desalted with a RP-C_{18} ZipTip. The 10 μl ZipTip pipette tip with a bed of chromatography media at the end ($\approx$ 490 μm inner diameter) was wetted with 50% methanol in water, then equilibrated with 0.1% TFA in water 3 times. The sample was aspirated into the ZipTip and dispensed out. This procedure was repeated 20-25 times for maximum extraction of the protein/peptide. The tip was washed with a washing solution consisting of 0.1% TFA in water 7 times. Elution solution (50% (v/v) methanol, 49% water and 1% acetic acid) was aspirated and dispensed through ZipTip several times, and then the ZipTip was set in front of the MS inlet for ESTASI-MS analysis. Analysis of five antibiotics mixture (their structure, molecular weight and the dissociation constant are presented in the Appendix 8.0) was also demonstrated with the ZipTip-ESTASI-MS.

3. Results and discussion

3.1 Electrostatic spray ionisation of peptide or protein from micropipette tip

The ESTASI procedure has been already discussed several times in the previous chapters. In the experiments with the micropipette tip, the electrode is placed around the tip and connected to the HV source to induce the spray. The outlet of the micropipette tip (≈ 650 μm inner diameter) is set 2-3 mm away from the mass spectrometer inlet. When the pulsed HV (0-9 kV) is applied, the spray of ions occurs for MS analysis.

In order to perform the spray from the tip by ESTASI-MS, the tip was filled with 5 μl of sample in 50% MeOH/49% H_2O/1% acetic acid. Using the current setup, the analysis of small molecules, peptides and proteins were performed with the examination of their limits of detection (LODs). Figure 8.2a shows the mass spectrum of caffeine (MW=194.19 g/mol) generated by ESTASI-MS, with a caffeine concentration of at most 10 ng/ml (51nM). In Figure 8.2b and 8.2c, the LODs were found to be 13 ng/ml (10 nM, S/N = 5) for angiotensin I and 70 ng/ml (6 nM, S/N = 10) for cytochrome c. For comparison, the LOD for the same peptide and protein performed by ESI with commercial ionization source was found to be at 45 to 50nM and 65 to 70 nM, respectively[16].

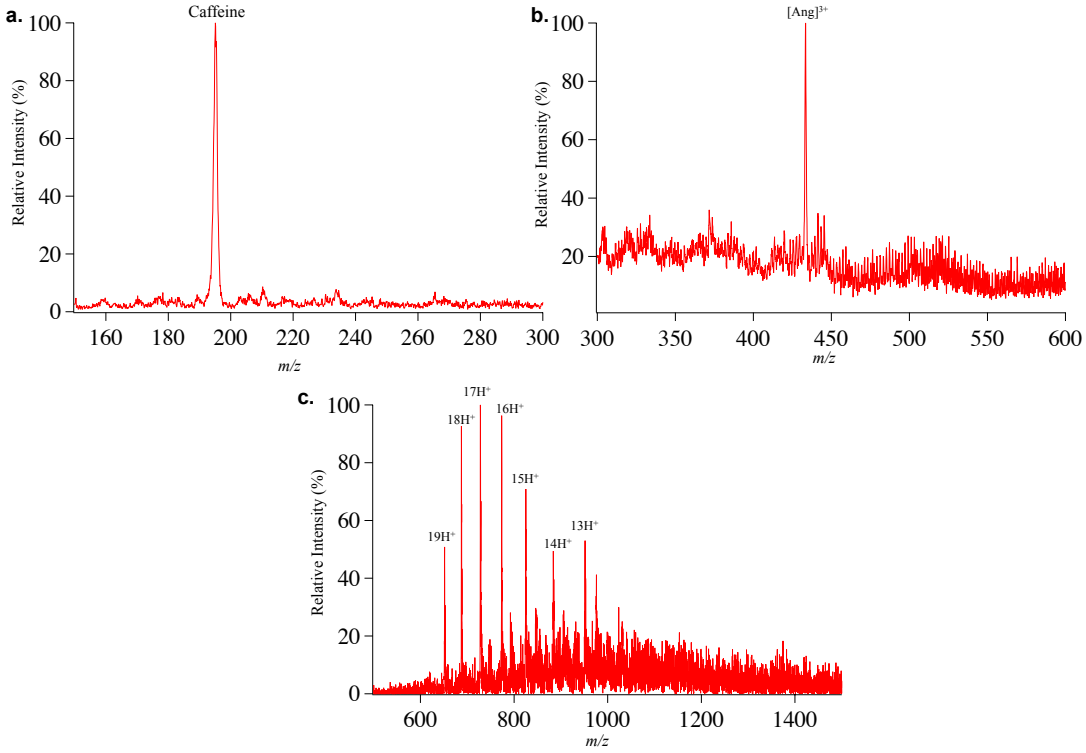


Figure 8.2. Mass spectra of **a.** caffeine 51 nM, **b.** angiotensin I 10 nM and **c.** cytochrome c 6 nM under positive ion mode. All the samples were prepared in ESI buffer (50% MeOH/49% H_2O/1% acetic acid). The ions were generated from the micropipette tip by ESTASI-MS by applying high potential of 9.0 kV.

3.2 Electrostatic spray ionisation of peptide or protein from RP-C_{18} ZipTip

The presence of salts in sample drastically influences the analyte signal of MS. In order to overcome this limitation, the ZipTip approach was applied in sample desalting followed with direct analysis by ESTASI-MS. To demonstrate the feasibility of using the proposed protocol as a fast and efficient way of salt-containing sample analysis, angiotensin and cytochrome c with different salt concentrations were desalted, and then detected directly by ESTASI-MS from the ZipTip. The commercial RP-C_{18} ZipTip® was wetted, equilibrated with 0.1% TFA in water, and then filled with aqueous solution of cytochrome c in 1M and 0.1M $NaCl^-$ for protein binding. Afterward 4 μl of elution buffer (50% (v/v) methanol, 49% water and 1% acetic acid) was added to the tip by a micropipette and the electrode was placed around the tip 0.7 mm away from the end of the tip. The HV source was connected to the electrode and a pulsed high voltage of 0-9.0 kV was used to generate the ions from the ZipTip.

As shown in Figure 8.3, the results achieved after desalting and direct ESTASI-MS from the tip was compared with the one obtained by ESI-MS with

commercially equipped ion source, and by normal ESTASI-MS where samples were deposited on a polymer plate for analysis. For ESI-MS and normal ESTASI-MS without desalting, no ions of cytochrome c were observed. However, using ZipTip-ESTASI-MS, the analysis was improved and multiple charged cytochrome c ions were observed: charge states +13, +18 in the presence of 1M NaCl and from +11 to +15 in 0.1M NaCl. It should be noted that the concentration of cytochrome c in all experiments was only 8 nM, quite close to the LOD of ESTASI-MS analysis with the current MS instrument and micropipette tip as emitter, indicating a very efficient desalting and high sample recovery, and furthermore a very good sensitivity of the current strategy.

Angiotensin I in the presence of salt was also analyzed by the ZipTip-ESTASI-MS. As for proteins, the results for peptides were compared with those obtained by ESI-MS and traditional ESTASI-MS. In Figure 8.4 I and II, the mass spectra of 38 nM angiotensin I with 0.1 M and 0.01M sodium chloride are presented. In the case of higher salt concentration, angiotensin ions were not observed before the peptide purification by ZipTip. On Figure 8.4 I.C $[M + 2H]^{2+}$ and $[M + 3H]^{3+}$ ions were detected by the ZipTip-ESTASI-MS. When the concentration of salt in the peptide sample was 0.01M NaCl, the angiotensin ions were recorded in all three cases. Ziptip-ESTASI-MS shows best detection results by considering the signal intensities and the simplicity of mass spectra. All the spectra were recorded under positive ion mode. The concentration of Ang I was also quite low, indicating a good sensitivity of this method in the analysis of peptide.

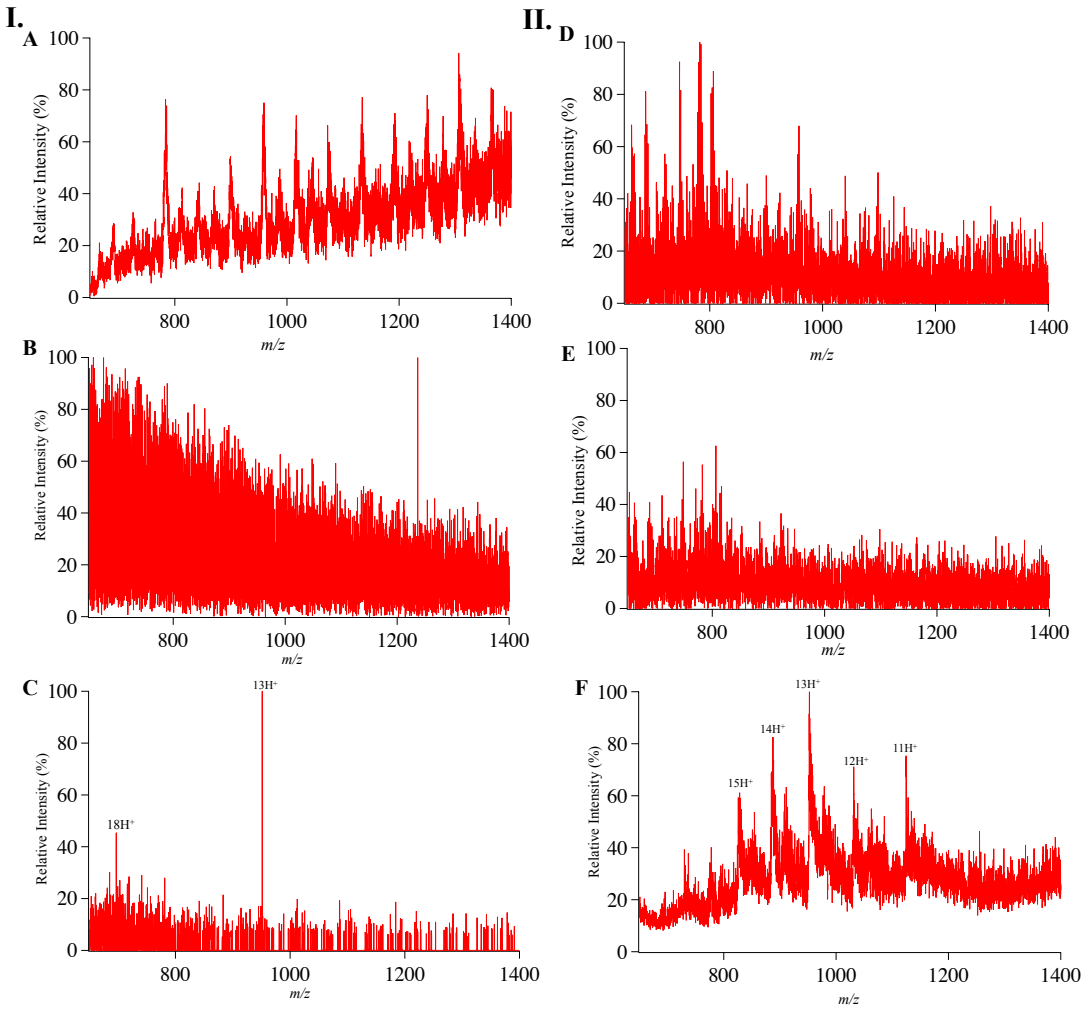


Figure 8.3. I. Mass spectra of 8nM cytochrome c with 1M $NaCl^-$ in ESI buffer obtained by **A)** ESI-MS with commercially equipped ESI source under direct-infusion mode; **B)** sprayed from insulating plate by ESTASI-MS, **C)** ZipTip-ESTASI-MS; **II.** 8 nM cytochrome C with 0.1 M $NaCl^-$ in ESI buffer determined by **D)** ESI-MS with commercially equipped ESI source under direct-infusion mode; **E)** sprayed from insulating plate by ESTASI-MS, **F)** ZipTip-ESTASI-MS. All the spectra were obtained in a positive ion mode.

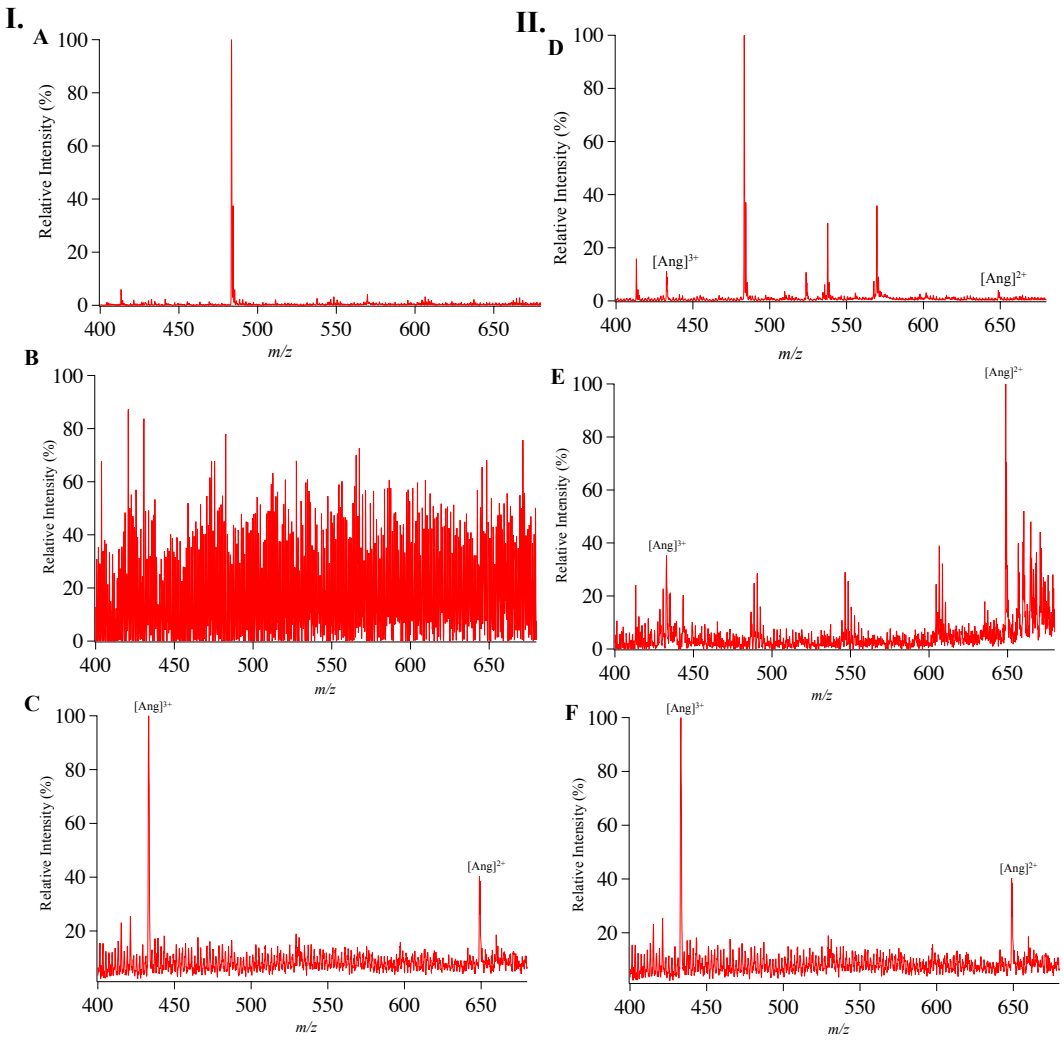


Figure 8.4. I. Mass spectra of 38 nM angiotensin I with 0.1M NaCl in ESI buffer and **II.** 38 nM angiotensin I with 0.01M NaCl in ESI buffer obtained by **A, D)** ESI-MS with commercially equipped ESI source under direct-infusion mode; **B, E)** sprayed from insulating plate by ESTASI-MS; **C, F)** ZipTip-ESTASI-MS. All the MS experiments were performed in a positive ion mode.

In addition to protein/peptide analysis by RP-C_{18} ZipTip-ESTASI-MS, the analysis of different antibiotics in the presence of salts was also realized. The mixture of five antibiotics, CIP, ENR, LOM, FLE and OFL 0.25 mg/ml each containing 10mM NaOH was diluted 2 times in 50% MeOH/49% H_2O/1% acetic acid, and used to illustrate the principle of coupling desalting procedure with ESTASI-MS analysis, as shown in Figure 8.5. Even though the salt concentration in the sample of antibiotics was small enough that the antiobiotics can be detected by ESTASI-MS without desalting, the ZipTip-ESTASI-MS strategy improved the ion detection and decreased the background noise, significantly. The mixture of antibiotics was spayed from the

polymer plate directly to L-shape MS inlet by depositing 5 μl of the solution on top of it and by applying 9.0kV. This result was compared with those detected after direct analysis of the mixture by ZipTip-ESTASI-MS. The results observed are quite similar, all five compounds of the sample were identified, however some random peaks corresponding to background peaks are presented on the spectra recorded before sample desalting by ZipTip.

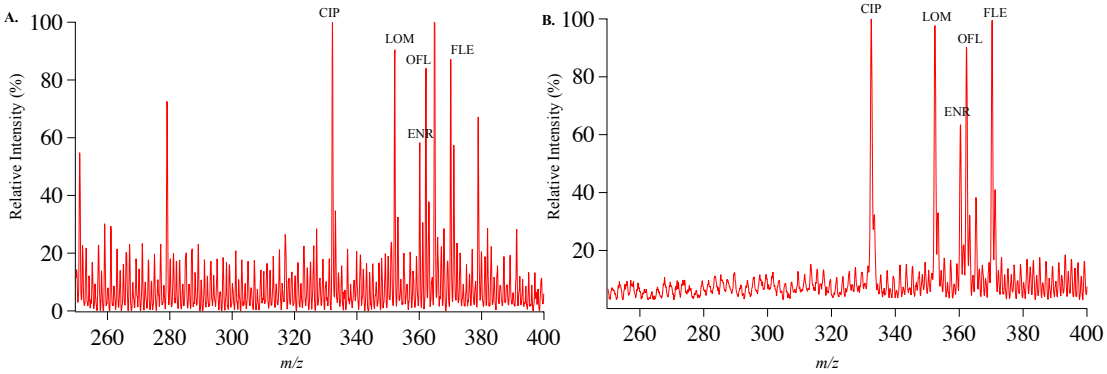


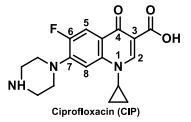
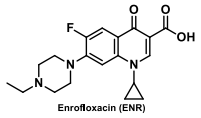
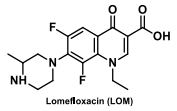
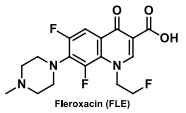
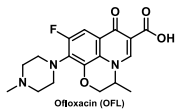
Figure 8.5. The mass spectra of antibiotics with 100mM NaOH in ESI buffer **A.** before desalting by ESTASI-MS from the polymer plate and **B.** after the desalting using RP-C_{18} ZipTip- ESTASI-MS. All the spectra were generated in a positive ion mode.

4. Conclusions

ESTASI-MS has been used for direct analysis of the samples from the disposal micropipette tip and RP-C_{18} ZipTip®. Protein/peptide sample containing different amounts of salt were analysed by ZipTip-ESTASI-MS and compared with traditional ESTASI-MS from the plate, and with ESI-MS. The method proposed could be used for sample desalting prior to MS analysis and drastically improving the ion detection. Using the micropipette tip as emitter, it is possible to decrease the experimental time and run high throughput analysis of biological samples. The proposed method is easy to use since most of the laboratories have all the equipment needed. Micropipette tips are in low cost, and the volume of sample required for analysis is low. The ESTASI-MS could be applied in numerous research fields where the biological sample cleanup is needed.

Appendix 8.0

The structures, molecular weight and dissociation constants of antibiotics

Fluoroquinolones	M.W.	pK_a1	pK_a2
 Ciprofloxacin (CIP)	331.34	5.90	8.89
 Enrofloxacin (ENR)	359.39	6.32	8.62
 Lomefloxacin (LOM)	351.35	5.82	9.30
 Fleroxacin (FLE)	369.34	5.46	8.00
 Ofloxacin (OFL)	361.37	5.97	8.28

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Chapter IX.

Summary and perspectives

1. Summary

This thesis has focused on developing new analytical tools for proteome analysis based on gel electrophoresis and mass spectrometry.

A homemade device for improving OFFGEL electrophoresis by applying a more homogeneous electric field was proposed and developed. Finite element modeling was used to show that the multi-electrode system provides indeed a more uniform electric field offering faster and better protein separations compared to a conventional two-electrode device. To fully test the multi-electrode system, a complex protein mixture was analyzed showing both an improved protein separation and an increase in protein collection efficiency in a shorter time.

Protein fractionation and desalting by OFFGEL electrophoresis with an immobilized ultra narrow pH gel in a three-well format has been proposed. This approach purifies complex samples with speed and simplicity prior to the application of other separation methods, as demonstrated with digested and non-digested *E. coli* protein extract. UV-Vis spectroscopy, MALDI-MS, SDS-PAGE and LC-MS/MS were employed for the quantitative and qualitative characterization of the separation results. The electrophoretic methodology was simulated by finite element methods.

Based on the electrophoretic separation method discussed in Chapter III, an electro-elution device was introduced to purify protein/peptide samples from an immobilized ultra narrow pH gradient gel. Extracted molecules can be directly deposited and analyzed by MALDI–MS, ESI-MS or quantification can be performed by UV-Vis spectroscopy. Preliminary results show that the electro-elution device is a fast and effective way to purify the sample of interest and to avoid time-consuming steps of extraction from the polyacrylamide gel.

Another topic developed and presented in this thesis is the new ambient ionization technique called ElectroStatic Spray Ionization (ESTASI) mass spectrometry. The proposed technique was used to ionize molecules from untreated samples on a surface. We have coupled gel electrophoresis to mass spectrometry by ESTASI. Nanogram samples loaded in the gel can be detected after electrophoretic separation, allowing the direct coupling of gel electrophoresis to mass spectrometry avoiding extra time-consuming steps such as gel staining and sample extraction procedures.

ESTASI was also applied to the analysis of perfume fragrances deposited on different types of paper. In order to make the detection of ions from the paper more effective the transfer capillary of the mass spectrometer was modified to an "L-shape". This strategy yields rapid characterization of the authentic fragrances, avoiding time-consuming sample-preparation steps, and thereby can help the perfume industry to perform quality checks in several seconds, as well as identify fraudulent perfumes.

ESTASI-MS was also used, as a quantitative technique for caffeine analysis. The amount of caffeine in different beverages was analyzed using a standard addition strip. The values achieved were compared with the ones obtained by a classic HPLC method reported by the manufacturer and were shown to be in good agreement. This strategy can be used for fast and high-throughput quantification of different compounds in samples of interest in many industrial fields.

The presence of salts in many biological samples often makes it impossible to analyze such samples by electrospray ionization mass spectrometry (ESI-MS). Here a new approach of coupling a RP-C_{18} ZipTip to ESTASI-MS has been demonstrated (Chapter VIII). Samples containing different concentrations of salts were desalted and directly sprayed into the inlet of a mass spectrometer. The spectra recorded were compared to those obtained by ESI-MS and by traditional ESTASI-MS from the plate. ZipTip-ESTASI-MS provides fast and effective sample cleaning procedures prior the MS detection.

At the end of a thesis new ideas are often generated; with this in mind, two main untested concepts were thereafter presented as a continuation of the research topics of this thesis. The developments of a new electro-elution device for high-throughput protein/peptide analysis, as well as the development of new two-dimensional gel electrophoresis strategy are discussed.

2. Electro-elution device for protein/peptide purification using ultra narrow pH gradient gels

In Chapter IV, a new electro-elution device for protein/peptide purification was proposed. The results presented are very promising, however, during the experiment we encountered several problems, including ineffective protein separation, capillary blocking and poor sample mixing. In order to make the device more effective for purification of complex mixtures, and to overcome the limitations presented above, a new configuration of the elution well was required. A revised setup geometry should solve such drawbacks as mixing of the sample in the elution well, capillary blocking, and to decrease the influence of dead volume on molecule detection (see Figure 9.1). Instead of having two wells for loading and collecting the samples, we proposed a straight microchannel allowing the liquid to pass through the channel without accumulating.

In order to keep the flow rate constant, the peristaltic pump should be changed to another pumping system. Blockage of the silica capillary by the re-swollen gel during pumping influenced the distance between proteins with similar migration speed, causing problems in ESI-MS detection. Introducing air bubbles into the capillary at specific intervals, as done previously in push-pull probes for SECM[1], was found to improve the separation and resolve two proteins between each other. To show that the electro-elution device can be used for high-throughput analysis purification of complex biological mixtures, the fractionation step should be repeated.

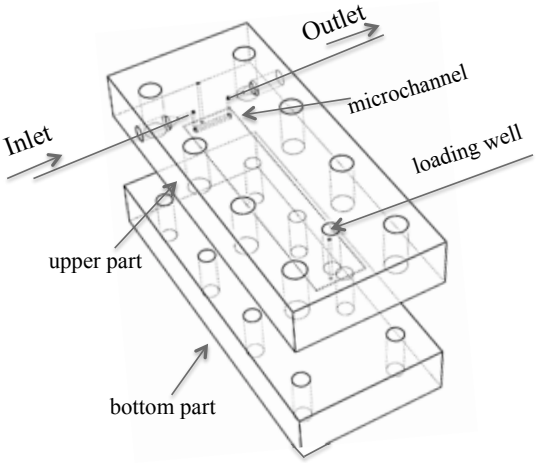

Figure 9.1. Proposed electro-elution device.

The electro-elution device can also be used as the second dimension after OFFGEL electrophoresis, where the molecules would be separated according to their p*I* values; afterwards, the recovered fraction of interest can be purified and directly analyzed by mass spectrometry.

Protein efficiency could also be improved by separating the samples using a constant pH media gel. As no commercialized gels with a constant pH are available, the preparation of homemade gels with constant pH was required. Moreover, it could allow for the separation of proteins that require a pH that is not readily available as commercial IPG gel strips.

Inkjet printing technologies, to print the constant pH gels on polymeric or paper films can be used. Inkjet printing technologies are based on the delivery of an ink droplet (*i.e.* 10 pL) with a high accuracy and reproducibility. Inkjet printing is a very powerful tool that has been adopted to print various functional materials, such as conductive inks, light emitting diodes, and even three-dimensional structures[2]. Using this technique it is possible to prepare polyacrylamide gels of different sizes, shapes and thicknesses, opening new doors to the format of gel electrophoresis.

3. Two-dimensional gel electrophoresis coupled to ESTASI-MS.

In the future, ESTASI-MS can be applied to the analysis of protein/peptide mixtures after two-dimensional gel electrophoresis (see Figure 9.2). As the first dimension, isoelectric focusing was utilized; whereby, the sample would be separated by p*I* values in a linear pH gradient gel. After separation, the molecules would be focused on the specific pH of the gel. The second-dimension took place in a constant pH media, where the sample will be separated according to their charge. To cast the gels with the constant pH media, inkjet printing technologies can be utilized. However, using inject technologies would be a challenging task, since parameters, such as viscosity and surface tension, drastically affect the printing efficiency, optimizing the gel casting can become exceedingly complex. A preliminary result shows that in order to avoid time-consuming steps of inject printing technologies; the gel of specific pH can be polymerized using an OmniCure® S2000 mercury UV lamp (Lumen Dynamics, Mississauga, Ontario, Canada). The stock solution is loaded into the compartment of appropriate size and length and the UV light, with intensity of 100%, is applied for 180 seconds to transform the liquid into a polymer.

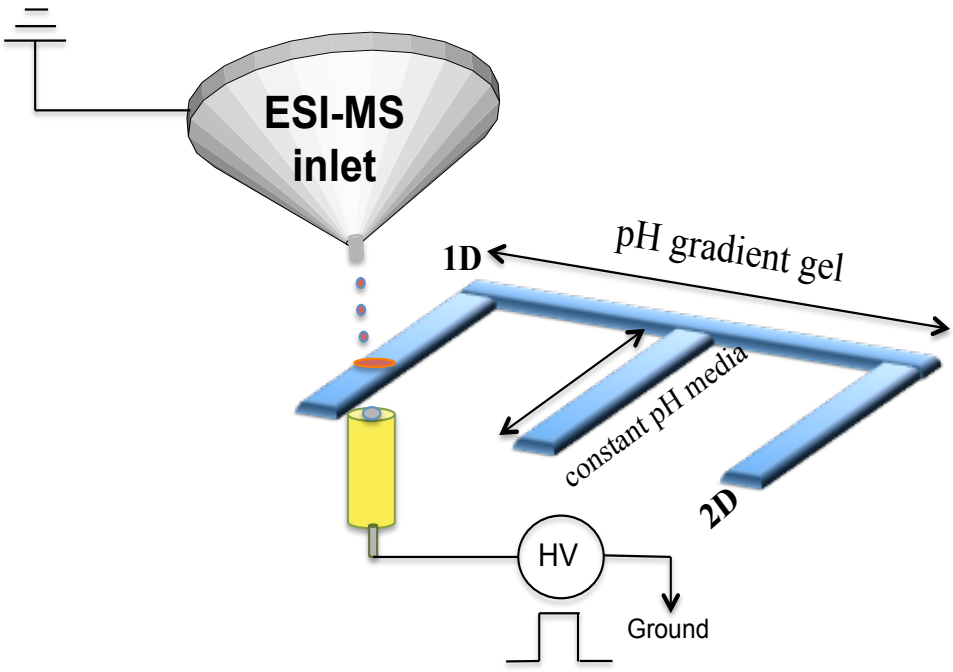

Figure 9.2. Schematically representation of two-dimensional gel electrophoresis coupled to electrostatic spray ionization mass spectrometry.

When the second dimension by the charge is completed, the gel strips can be analyzed by ESTASI-MS. The electrode would be placed behind the gel area of interest and a high voltage would be applied to the electrode, for generating the ions from the surface, which will directly enter the mass spectrometer inlet.

2D gel electrophoresis followed by ESTASI-MS can be used in top-down proteomics, for analysis of intact proteins and its PTMs. Coupling these two techniques would increase protein sequence coverage and decrease the experimental time.

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CURRICULUM VITAE

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Journal Publications:

1. *E. Tobolkina*, F. Cortés-Salazar, D. Momotenko, Julien Maillard, Hubert H. Girault, Segmented Field OFFGEL® Electrophoresis, Electrophoresis, vol. 33, p. 3331-3338, 2012.
2. Liang Qiao, Romain Sartor, Natalia Gasilova, Yu Lu, *Elena Tobolkina*, Baohong Liu, Hubert H. Girault, Electrostatic-Spray Ionization Mass Spectrometry, Analytical Chemistry, vol. 84, num. 17, p. 7422-7430, 2012.
3. *Elena Tobolkina*, Liang Qiao, Guobin Xu, Hubert H. Girault, Electrostatic Spray Ionization Mass Spectrometry Sniffing for perfume fingerprinting, Rapid Communications in Mass Spectrometry, vol. 27, num. 21, p. 2310–2316, 2013.
4. *Elena Tobolkina*, Liang Qiao, Baohong Liu and Hubert H. Girault, Coupling Gel Electrophoresis to Mass Spectrometry by Electrostatic Spray Ionisation, Analytical Chemistry, 85 (9), 4745-4752, 2013.
5. Liang Qiao, *Elena Tobolkina*, Andreas Lesch, Baohong Liu and Hubert H. Girault, Electrostatic Spray Ionization Mass Spectrometry Imaging, Analytical Chemistry, 86 (4), 2033-2041, 2014
6. *Elena Tobolkina*, Fernando Cortés-Salazar, Liang Qiao, Hubert H. Girault, "Proteins/peptides purification by a three-well OFFGEL electrophoresis with immobilized ultra narrow pH gradient gels", Analytical Methods, 2014, 6 (12), 3995-4002.
7. *Elena Tobolkina*, Liang Qiao, Hubert H. Girault Standard addition strip for quantitative electrostatic spray ionization mass spectrometry analysis: Determination of caffeine in drinks., accepted in Talanta, 2014, vol. 130, pp. 377-381.

Conferences:

- 5th Summer Coarse on Mass Spectrometry in Biotechnology and Medicine, Dubrovnik, Croatia (2011)
- 27th International Symposium on MicroScale Bioseparations and Analyses, Geneva, Switzerland (2012), Poster presentation: "Multi-electrode setup for protein electrophoresis".
- 61st American Society for Mass Spectrometry, Minneapolis, United States (2013), Oral presentation: "Coupling Gel Electrophoresis to Mass Spectrometry by Electrostatic Spray Ionization".
- Mass spectrometry meeting "SGMS" (2013), Poster presentation: Electrostatic Spray Ionization Mass Spectrometry, Beatenberg, Switzerland.

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Physical chemistry
Laboratory of liquid electrolytes, Department of Chemistry, Saint-Petersburg State University (SpBSU), Russia

Diploma Thesis title: Spectrophotometric study of lanthanide series of metalloporphyrins

Research experience: Four individual research projects (2005 – 2009, total 12 month)
Diploma research project (2009, 7 month)

Conferences:

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- International conference for students and graduated students, SpBSU, Russia (2010) Poster session: "Spectroscopy of lanthanide complexes with tetraphenylporphyrins".

Chemistry Skills

- Proficient with most analytical techniques, including spectroscopic methods (atomic absorption and emission spectroscopies, UV-Vis spectrophotometry, X-ray fluorescence), MALDI-MS, ESI-MS, electrophoretic and chromatographic separation methods (OFFGEL electrophoresis, capillary electrophoresis, gel electrophoresis, high performance liquid chromatography (HPLC), gas chromatography (GS)).
- Experienced to work under clean room environment.
- Practiced user of COMSOL Multiphysics (vers. 3.5a) finite element package.

CURRICULUM VITAE

- An experienced user of Mathematica, IGOR PRO, ChemDraw, Microsoft Office, Operating Systems (MacOS, Windows, Linux), Sketch Up.

Experience

Analytical chemistry teacher from 2010-2013 (more than 630 academic hours of teaching experience)

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Skills

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