

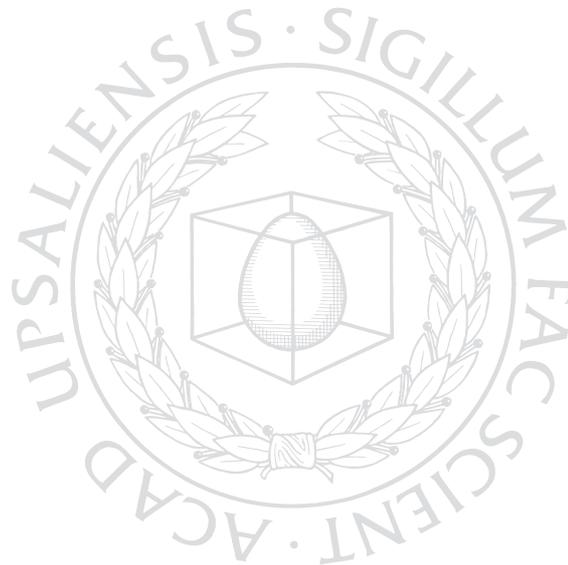


UPPSALA
UNIVERSITET

*Digital Comprehensive Summaries of Uppsala Dissertations
from the Faculty of Science and Technology 31*

Analysis of Complex Biological Samples using Liquid Chromatography-Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

MARGARETA RAMSTRÖM



ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2005

ISSN 1651-6214
ISBN 91-554-6198-0
urn:nbn:se:uu:diva-5729

Dissertation presented at Uppsala University to be publicly examined in Room B22, BMC, Uppsala, Wednesday, May 4, 2005 at 10:15 for the degree of Doctor of Philosophy. The examination will be conducted in English.

Abstract

Ramström, M. 2005. Analysis of Complex Biological Samples using Liquid Chromatography-Fourier Transform Ion Cyclotron Resonance Mass Spectrometry. Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology* 31. 62 pp. Uppsala. ISBN 91-554-6198-0.

Studies of protein and peptide expression are vital in order to understand complex biological systems. As demonstrated in this thesis, on-line packed capillary liquid chromatography-Fourier transform ion cyclotron resonance mass spectrometry (LC-FTICR MS) is a useful analytical tool for such studies.

A proteomics method, based on global tryptic digestion and subsequent separation and detection of the peptides by LC-FTICR MS, was developed for qualitative analysis of body fluids. Initial experiments on cerebrospinal fluid (CSF) provided results that were comparable or superior to those achieved by more time- and sample-consuming techniques. The method was also successfully applied on plasma and amniotic fluid. One of the major challenges in proteomics is the broad dynamic range of proteins in biological matrices. The advantages of removing high-abundant components from CSF and plasma prior to MS were demonstrated.

In order to search for potential biomarkers, mass chromatograms of CSF from patients suffering from amyotrophic lateral sclerosis (ALS) and controls were compared using an in-house constructed pattern recognition program. ALS-specific patterns were observed, and four out of five unknown samples were correctly assigned. Alternative strategies to quantitatively compare two pools of samples rely on differential chemical labeling. The performance of one such method, quantification-using-enhanced-signal-tags, was investigated in complex sample analysis. The experimental intensity ratios were proven to be consistent with the prepared concentration ratios of abundant proteins in CSF.

Finally, the thesis reports on the first experiments where electron capture dissociation (ECD) was successfully incorporated in on-line LC-MS experiments. ECD and nozzle-skimmer fragmentation were applied to a sample of endocrine peptides extracted from mouse pancreatic islets. The two fragmentation methods provided complementary information. However, the method needs further optimization before it can be applied in the analysis of more complex samples, such as body fluids.

Keywords: mass spectrometry, liquid chromatography, protein, proteomics, peptide, Fourier transform ion cyclotron resonance mass spectrometry, cerebrospinal fluid, amyotrophic lateral sclerosis, electrospray ionization, electron capture dissociation

Margareta Ramström, Department of Chemistry, Department of Analytical Chemistry, Box 599, Uppsala University, SE-75124 Uppsala, Sweden

© Margareta Ramström 2005

ISSN 1651-6214

ISBN 91-554-6198-0

urn:nbn:se:uu:diva-5729 (<http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-5729>)

To Mats

List of Papers

- I **M. Ramström**, M. Palmblad, K.E. Markides, P. Håkansson and J. Bergquist, "Protein identification in cerebrospinal fluid using packed capillary liquid chromatography Fourier transform ion cyclotron resonance mass spectrometry", *Proteomics*, Vol. 3 (2003), No. 2, 184-190.
- II **M. Ramström**, I. Ivonin, A. Johansson, H. Askmark, K.E. Markides, R. Zubarev, P. Håkansson, S.-M. Aquilonius and J. Bergquist, "Cerebrospinal fluid protein patterns in neurodegenerative disease revealed by liquid chromatography-Fourier transform ion cyclotron resonance mass spectrometry", *Proteomics*, Vol. 4 (2004), No. 12., 4010-4018.
- III S. Nilsson, **M. Ramström**, M. Palmblad, O. Axelsson and J. Bergquist, "Explorative Study of the Protein Composition of Amniotic Fluid by Liquid Chromatography Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry", *J. Proteome Res.*, Vol. 3 (2004), No. 4, 884-889.
- IV **M. Ramström**, C. Hagman, J.K. Mitchell, P.J. Derrick, P. Håkansson and J. Bergquist, "Depletion of High-Abundant Proteins in Body Fluids Prior to Liquid Chromatography Fourier Transform Ion Cyclotron Resonance Mass Spectrometry", *J. Proteome Res.*, Vol. 4 (2005), published on Web 05 Feb.
- V C. Hagman, **M. Ramström**, P. Håkansson and J. Bergquist, "Quantitative Analysis of Tryptic Protein Mixtures Using Electrospray Ionization Fourier Transform Ion Cyclotron Mass Spectrometry", *J. Proteome Res.*, Vol. 3 (2004), No. 3, 587-594.
- VI C. Hagman, **M. Ramström**, M. Jansson, P. James, P. Håkansson and J. Bergquist, "Reproducibility of Tryptic Digestion Investigated by Quantitative Fourier Transform Ion Cyclotron Resonance Mass Spectrometry", *J. Proteome Res.*, Vol. 4 (2005), published on Web 05 Feb.

- VII M. Palmblad, Y.O. Tsybin, **M. Ramström**, J. Bergquist and P. Håkansson, "Liquid chromatography and electron-capture dissociation in Fourier transform ion cyclotron resonance mass spectrometry", *Rapid Commun. Mass Spectrom.*, Vol. 16 (2002), No. 10, 988-992.
- VIII **M. Ramström**, C. Hagman, Y.O. Tsybin, K.E. Markides, P. Håkansson, A. Salehi, I. Lundquist, R. Håkanson and J. Bergquist, "A novel mass spectrometric approach to the analysis of hormonal peptides in extracts of mouse pancreatic islets", *Eur. J. Biochem.*, Vol. 270 (2003), No. 15, 3146-3152.

Reprints were made with kind permission from the publishers.

Author Contribution

I was responsible for planning and performing the experiments in **Paper I**. I wrote the paper and took active part in interpretation of the results. My contribution in **Paper II** was to plan and perform the mass spectrometry-based experiments, to take part in data analysis and to write the paper. In **Paper III**, the experimental work was performed in collaboration with S. Nilsson, while M. Palmblad was responsible for the data-evaluation. We discussed the results together. The ideas behind **Papers IV** were mostly mine; I performed the experiments with skilful assistance from C. Hagman and J. Mitchell and I wrote the paper. I constructed some of the programs used for data analysis in **Papers V** and **VI**, and participated in running the experiments and evaluation of the results. In **Paper VII**, I took part in sample preparation, running the experiment and discussions of the results. I was responsible for planning and performing the experiments in **Paper VIII**, for interpretation of the results and for writing the article.

Related Papers Not Included in the Thesis

- **M. Ramström**, M. Palmblad, A. Amirkhani, Y.O. Tsybin, K.E. Markides, P. Håkansson and J. Bergquist, "Micro-capillary liquid chromatography Fourier transform ion cyclotron resonance mass spectrometry - a powerful tool for peptide and protein identification", *Acta Biochimica Polonica*, Vol. 48, No. 4 (2001), 1101-1104.

- M. Palmblad, **M. Ramström**, K.E. Markides, P. Håkansson and J. Bergquist, "Prediction of Chromatographic Retention and Protein Identification in Liquid Chromatography/Mass Spectrometry", *Anal. Chem.*, Vol. 74, No. 22 (2002), 5826-5830.
- J. Buijs, **M. Ramström**, M. Danfelter, H. Larsericsdotter, P. Håkansson and S. Oscarsson, "Localized changes in the structural stability of myoglobin upon adsorption onto silica particles, as studied with hydrogen/deuterium exchange mass spectrometry", *J. Colloid Interface Sci.*, Vol 263 (2003), No. 2, 441-448.
- S.-L. Wu, G. Choudhary, **M. Ramström**, J. Bergquist and W.S. Hancock, "Evaluation of Shotgun Sequencing for Proteomic Analysis of Human Plasma Using HPLC Coupled with Either Ion Trap or Fourier Transform Mass Spectrometry", *J. Proteome Res.*, Vol. 2 (2003), No. 4, 383-393.
- M. Palmblad, **M. Ramström**, C.G. Bailey, S.L. McCutchen-Maloney, J. Bergquist and L.C. Seller, "Protein identification by liquid chromatography-mass spectrometry using retention time prediction", *J. Chromatogr. B*, Vol. 803 (2004), No. 1, 131-135.
- **M. Ramström** and J. Bergquist, "Miniaturized proteomics and peptidomics using capillary liquid separation and high resolution mass spectrometry", *FEBS Letters*, Vol. 567 (2004), No. 1, 92-95.
- Y.O. Tsybin, **M. Ramström**, M. Witt, G. Baykut and P. Håkansson, "Peptide and protein characterization by high-rate electron capture dissociation Fourier transform ion cyclotron resonance mass spectrometry", *J. Mass Spectrom.*, Vol. 39 (2004), No. 7, 719-729.

Contents

1. Introduction.....	13
2. Mass Spectrometry and Liquid Chromatography	15
Mass Spectrometry.....	15
Electrospray Ionization.....	16
Fourier Transform Ion Cyclotron Resonance Mass Spectrometry	17
Miniaturized Liquid Chromatography.....	20
On-line LC-FTICR MS	21
Experimental Setup	21
3. Qualitative Proteomics.....	24
Expression Proteomics Methodology.....	24
FTICR MS in Qualitative Proteomics	25
Packed Capillary LC-FTICR MS of Body Fluids	26
Human Plasma.....	26
Cerebrospinal Fluid	27
Amniotic fluid.....	27
Experimental Procedure.....	28
Identification of Proteins in Cerebrospinal Fluid	29
A General Method for Identification of Proteins in Body Fluids	31
Removal of High-Abundant Proteins in Body Fluids.....	31
4. Comparative Proteomics.....	34
Methods for Comparative Proteomics.....	34
Development of a Method for Comparative Proteomics using LC-FTICR MS	35
Amyotrophic Lateral Sclerosis	35
Description of the Pattern Recognition Program	35
Evaluation of the Results	37
5. Quantitative Proteomics using Chemical Labeling.....	39
Labeling Techniques	39
Quantification Using Enhanced Signal Tags.....	41
QUEST-markers for the Quantification of Proteins in Body Fluids	43
6. On-line Electron Capture Dissociation and LC-FTICR MS	45
Peptide Fragmentation.....	45

Collision-Induced Dissociation	46
Electron Capture Dissociation	47
ECD Combined with On-line LC-FTICR MS	47
The Method Applied to Peptides in a Tissue Extract	48
7. Conclusions and Future Aspects	51
8. Acknowledgements	53
9. Summary in Swedish	55
Analys av komplexa biologiska prover med vätske-kromatografi och Fouriertransform- joncyklotron-resonans-masspektrometri	55
Inledning	55
Analysmetoden	55
Tillämpningar och metodförbättringar	56
Slutsatser	57
10. References	58

Abbreviations

2D-PAGE	Two-dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis
AF	Amniotic fluid
ALS	Amyotrophic lateral sclerosis
BSA	Bovine serum albumin
CE	Capillary electrophoresis
CID	Collision-induced dissociation
ECD	Electron capture dissociation
ESI	Electrospray ionization
FTICR MS	Fourier transform ion cyclotron resonance mass spectrometry
HSA	Human serum albumin
ICAT	Isotope-coded affinity tags
LC	Liquid chromatography
<i>m/z</i>	Mass-to-charge
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
QUEST	Quantification using enhanced signal tags
RPLC	Reversed-phase liquid chromatography
SMTA	S-methyl thioacetimidate
SMTTP	S-methyl thiopropionimidate

1. Introduction

Proteins and peptides play indispensable roles in all living organisms. Consequently, studies of these molecules are important in many different research disciplines, including biochemistry, biology, medicine and nutrition. To better understand a complex biological system, it is of interest to characterize its proteome, i.e., all proteins expressed in the cell, the organism or the tissue under study. The terms proteome and proteomics were coined in 1994 by an Australian post doctoral student¹ and since then the field of proteomics has grown dramatically. However, it is worth mentioning that a project aiming at mapping all human proteins was proposed more than twenty years earlier²⁻⁴. It was suggested that information on, for example, protein function, subcellular localization and concentration as well as the position of the encoding gene should be listed in what was referred to as the Human Protein Index⁴. Important technical shortcomings and lack of funding at that time prevented this project from continuing^{1, 5, 6}. Instead, novel efficient methods for large-scale DNA sequencing were developed, and reports on the complete human genome were published in 2001^{7, 8}.

Improved knowledge of genomes has encouraged further investigation of the proteins, the actors in the biological system. A proteome is, in contrast to a genome, not constant over time, but varies with, e.g., age and disease state. One gene may give rise to several proteins after splicing and the gene products are often modified after translation. A careful investigation of a proteome involves the identification and quantification of proteins in the biological sample. Information on protein structures, functions and interactions are also necessary in order to fully characterize a proteome. Hence, proteomic research can be divided into several subdisciplines, for example expression proteomics, functional proteomics and structural proteomics^{6, 9}.

Method development in proteomics is an important and challenging task. Ideally, an experiment should yield maximum accurate information in the shortest possible time, consuming a minimal amount of sample. The method should also be reproducible and easy to automate. Mass spectrometry is today one of the most powerful tools in proteomics. Accurate mass information can, in combination with complementary methods, be used in most proteomics disciplines.

The aim of this thesis is to describe and discuss method development and novel aspects of how to use packed capillary liquid chromatography Fourier transform ion cyclotron resonance mass spectrometry (LC-FTICR MS) for

the analyses of complex protein and peptide samples. The thesis is based on eight papers, referred to as **Papers I-VIII**, focusing on proteomics studies of body fluids and on-line fragmentation of peptides. Methods have been developed for the identification and quantification of proteins in complex mixtures and for the comparison of protein patterns. Chapter 2 describes the principles behind the instruments and techniques used for the generation of all experimental data. The subsequent sections provide overviews of the covered fields of research, discuss the results obtained in the papers and put them into a global context.

2. Mass Spectrometry and Liquid Chromatography

Liquid chromatography (LC) and mass spectrometry (MS) are two of the most commonly applied analytical tools in biomolecular studies. When combined, MS should be regarded not solely as a sensitive detector for analytes separated by LC, but also as a complementary separation technique. In the chromatographic step, molecules are separated based on affinity for the stationary phase, while mass spectrometers constitute a group of instruments that separate the analytes with respect to mass-to-charge ratio.

Mass Spectrometry

The main parts of a mass spectrometer are the *ion source*, where the molecules are ionized, the *mass analyzer*, where molecules are separated according to their mass-to-charge (m/z) ratio and the *detector*, where the ion signal is transferred to qualitative and quantitative data. There are several different methods and principles for ionization, mass analysis and detection, and summaries of these can be found elsewhere^{10,11}. The two dominating ionization techniques in protein and peptide research are matrix assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). Both methods are soft techniques that allow for the subsequent mass analysis of intact macromolecules. Some of the most commonly used mass analyzers and information on their working principles are given in Table 1.

In this thesis, electrospray ionization in combination with FTICR mass spectrometry has been the analytical method of choice. Hence, these two techniques deserve more thorough descriptions.

Table 1. Three commonly used mass analyzers and short explanations of their working principles.

Instrument	Principle
Electromagnetic sector	<ul style="list-style-type: none"> • The ions are separated under the influence of combined electric and magnetic fields. • A mass spectrum is achieved by scanning the magnetic or electric fields.
Quadrupole	<ul style="list-style-type: none"> • Four parallel bars produce a combined DC electric and radio frequency field, which will allow for ions of a certain m/z to reach the detector. • A mass spectrum is achieved by scanning the fields.
Time-of-Flight	<ul style="list-style-type: none"> • The ions are accelerated in an electric field to achieve the same kinetic energy. • The velocities after acceleration depend on m/z. Hence, the time-of-flight to the detector depends on the m/z.

Electrospray Ionization

ESI is a very soft ionization technique in that it permits ionization of molecules without simultaneous ion fragmentation. Macromolecules, e.g., peptides and proteins can be analyzed and it is rather straightforward to combine on-line LC with ESI. While Dole and colleagues reported, in the 1960's¹², on the possibility to electrospray macromolecules, the first successful and competitive experiments where ESI was used as a mass spectrometric ion source were described in the mid 1980's by Fenn and coworkers¹³. For his work, J.B. Fenn was awarded the Nobel Prize in Chemistry in 2002.

A schematic drawing of the electrospray process is given in Figure 1. To generate an electrospray, a voltage of typically 2-3 kV is applied between the spray emitter and the inlet of the mass spectrometer. The distance between the capillary tip and the MS inlet is typically a few millimeters, and hence a very high electric field is obtained at the capillary tip. Electrophoretic movement will be induced in the liquid and capillary tip. If the applied electrical field is high enough, a so-called Taylor cone is formed and a jet emerges which breaks up into small droplets. The formed droplets will shrink due to solvent evaporation, while the charge will remain constant. Hence, the electrostatic repulsion will increase. When the charge-to-radius

relation approaches the Rayleigh limit, the droplets will undergo fission, i.e., be divided into smaller droplets. This procedure will be repeated until very small droplets remain. The actual formation of gas-phase ions from small droplets is debated, and two different mechanisms have been proposed; the *charged residue model* and the *ion evaporation model*. The former model suggests that very small droplets, containing only one charged ion, are

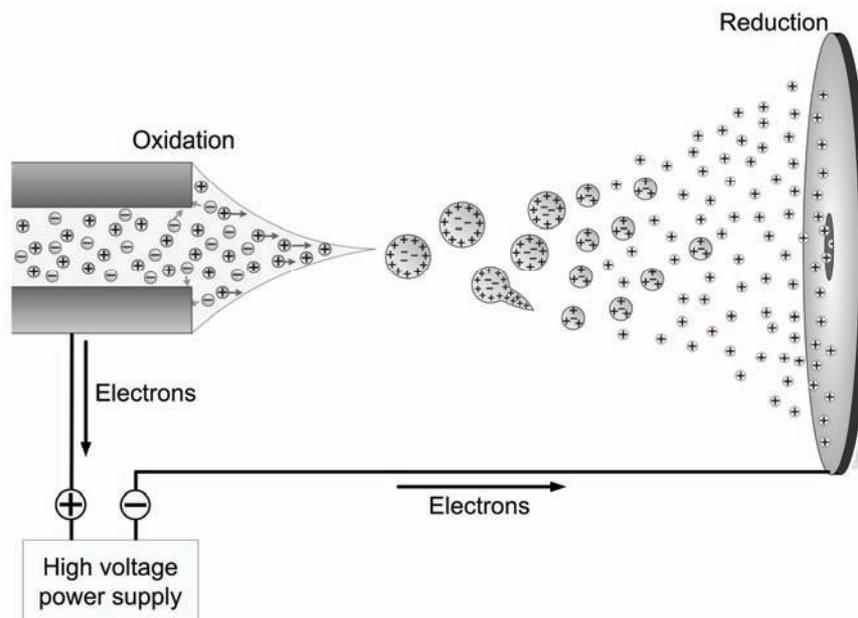


Figure 1. The electro spray process in positive mode. Electrophoretic movement of analytes is induced by the application of a high electric field between the electro-spray emitter tip and the inlet of the mass spectrometer. A fine jet is formed from the emitter tip, which breaks up into small charged droplets. Due to solvent evaporation and subsequent droplet fission caused by charge repulsion, gas-phase ions are eventually formed.

formed. Upon solvent evaporation, the ion is brought into the gas-phase¹². The ion evaporation model proposes that after the droplets have reached a certain radii (~10 nm), direct ion emission from the droplets becomes possible^{14, 15}. Several studies have been conducted in order to rule out what process dominates for different kinds of ions. It is now believed that the mechanism is dependent on, e.g., the ion size¹⁶.

Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

The Fourier transform ion cyclotron resonance mass spectrometer is a mass spectrometer of extraordinary resolution, mass accuracy and sensitivity.

Baseline resolution of two heptapeptides, differing in mass by 0.0005 Da, have been reported using a 9.4 T FTICR MS¹⁷. The mass measurement errors are typically on the low ppm- or sub-ppm-level and mass spectra have been recorded, consuming only 140 zmol of standard proteins¹⁸. FTICR MS is therefore a useful instrument for studies of many different analytes and samples. The principle of ion cyclotron resonance was incorporated into a mass spectrometer already in the 1950s^{19,20}, while the Fourier transform was first involved in the technique in 1974 by Comisarow and Marshall²¹. This marriage dramatically decreased the time required for generating a mass spectrum.

There are several different designs of FTICR mass spectrometers, commercially available and built in-house. The four major components common to all instruments are *a strong magnet, an analyzer cell, a pump system* to provide ultra-high vacuum, and a *sophisticated data system*²². The heart of the mass spectrometer is the analyzer cell, which is placed inside the strong, uniform and unidirectional magnetic field provided by the magnet. In this compartment, the ions are separated, trapped and detected.

Ion Motion in the Analyzer Cell

Cyclotron Motion

An ion of charge q , mass m and velocity v , moving in a uniform magnetic field (B) is subject to a force (F) as described by Equation 1.

$$\vec{F} = m \frac{d\vec{v}}{dt} = q\vec{v} \times \vec{B} \quad (1)$$

The force is perpendicular both to the magnetic field and the velocity, and the particle will therefore perform a circular motion, as illustrated in Figure 2. Let v_{xy} be the velocity component perpendicular to the magnetic field. The angular velocity is hence described by

$$\frac{dv_{xy}}{dt} = \frac{v_{xy}^2}{r} \quad (2)$$

and Equations 1 and 2 can be combined to yield

$$\frac{v_{xy}^2}{r} = qv_{xy}B \quad (3)$$

By definition, the angular velocity, ω , equals the velocity in the xy plane divided by the radius of the orbital motion, and Equation 3 can thus be simplified and rearranged to,

$$\omega = \frac{q}{m} B \quad (4)$$

Equation 4 is also known as the cyclotron equation. By measuring the frequencies, m/q can be determined. Of importance is that the cyclotron frequency, $2\pi\omega$, is the same for all ions of a given mass-to-charge ratio, regardless of their velocities (or kinetic energies). This is one of the main reasons why ultra-high resolution can be achieved in an FTICR MS. Also, frequency is one of the experimental parameters that can be determined with the highest accuracy.^{22, 23}

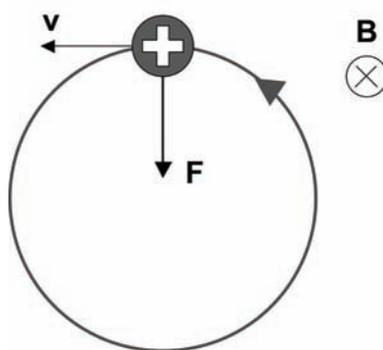


Figure 2. Cyclotron motion of a positively charged ion moving in a magnetic field, directed into the paper. The ion will experience a force perpendicular to the plane defined by the velocity and the magnetic field, and the ion will perform a circular motion.

Trapping and magnetron motion

The motion of ions in the analyzer cell is not only affected by the magnetic field. In order to extend the time for ions in the cell, and hence their detectability, a trapping electrical field is applied via the end cap electrodes perpendicular to the magnetic field axis. Positively charged ions will be stored in the trap if a positive potential is applied to both endplates. The combination of the electric and magnetic field also introduces a third motion, the magnetron motion.

Ion Excitation and Detection

The analyzer cell is equipped with a pair of plates for excitation of ions and another pair for the detection of ions (Figure 3). If a radio frequency signal is applied between the excitation plates, ions of the corresponding cyclotron frequency will absorb energy and spiral outwards. All ions of the same cyclotron frequency are excited coherently, and the group of ions induces an image current when passing the detection plates. In FTICR MS, ions of

many mass-to-charge ratios can be detected simultaneously. Broadband excitation is applied, e.g., by performing a rapid frequency sweep. The resulting image current is then Fourier transformed to give the frequencies and the corresponding mass-to-charge ratios.

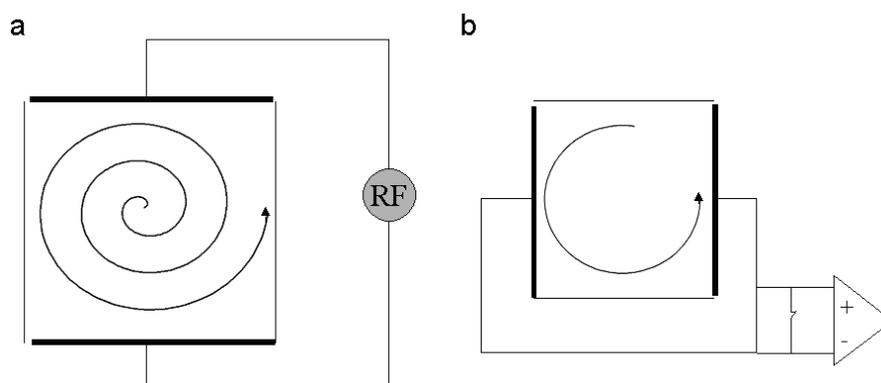


Figure 3. Ion excitation (a) and detection (b) in the analyzer cell. A radio frequency pulse is applied to the excitation plates. Ions which are in resonance with the excitation frequency will absorb energy and spiral outwards. The excited ions will induce an image current on the detector plates, and the signal is Fourier transformed to yield m/z of the ions.

Miniaturized Liquid Chromatography

Analysis of complex samples requires the introduction of an efficient separation step prior to mass spectrometric detection. One of the most powerful separation methods for peptides is liquid chromatography. Conventional LC-experiments are performed at flow rates on the mL/min scale, on columns of inner diameters of 2.1-4.6 mm. To meet specific demands, miniaturized LC systems have been developed²⁴. The obvious advantage associated with miniaturization is the ability to analyze smaller sample volumes at lower volumetric flow rates^{25,26}. Besides the lower solvent consumption, the miniaturized flow rate also allows for on-line combination of LC with ESI. Many solvents commonly used as mobile phases in LC are compatible with ESI. However, volatile buffers should be chosen in order to obtain stable electrospray conditions. Downscaling of the LC-system leads to increased sensitivity using a concentration sensitive detector. The terms used for miniaturized liquid chromatography might be confusing. According to a commonly used nomenclature, micro LC is performed on columns of inner diameters of

0.5-1.5 mm, capillary LC on 150-500 μm columns and nanoscale LC on 10-150 μm columns²⁶.

On-line LC-FTICR MS

A complication when combining on-line LC with FTICR MS was previously the relatively low duty cycle of the mass spectrometer. In early instruments, trapping and cooling of ions were typically achieved by pulsing gas into the analyzer cell. This procedure and the subsequent gas removal could take up to 1 min²⁷, which was incompatible with the elution times in LC. However, the first results on LC-ESI coupled on-line to a commercial FTICR MS were reported by Stacey *et al.*²⁷. In their approach, an alternative cell design²⁸ allowed for ion accumulation without gas-pulsing. A general solution to the problem concerning the low duty-cycle has been to separate the compartments for accumulation and detection spatially in the mass spectrometer. For example, external accumulation of ions in octupoles or hexapoles prior to injection into the analyzer cell, allows for duty cycles of nearly 100%. The benefits of combining on-line LC with external ion accumulation in FTICR MS were demonstrated by Senko *et al.*²⁹. This article was also the first one to report the combination of nanoscale LC with FTICR MS, which represents another important breakthrough in this field. Low femtomole³⁰ to subfemtomole³¹ detection limits of peptides were further reported using nanoscale LC. Today, the combination of miniaturized LC with FTICR MS is a more established tool for efficient analyses of complex samples, e.g., smaller proteomes³²⁻³⁴. Also, the possibility to integrate on-line fragmentation on the time-scale of the LC-experiments offers many advantages. These two aspects will be described and discussed in detail in the following chapters.

Experimental Setup

The experiments presented in this thesis (**Papers I-VIII**) were all performed on a Bruker Daltonics (Billerica, MA) BioAPEX-94e 9.4 T FTICR mass spectrometer. This instrument was installed in Uppsala in 1997, and has been described in detail by Palmblad *et al.*³⁵. Figure 4 gives an overview of the different parts of the instrument. The magnet is a 9.4 T superconducting magnet (Nb_3Sn) with passive shielding. The analyzer cell is of cylindrical shape, and contains one pair of detection plates and one pair of excitation plates. Although the mass spectrometer is equipped with ion sources for MALDI, electron impact, chemical ionization and secondary ion mass spectrometry, all experiments were conducted using electrospray ionization. The

electrospray interface was modified in-house. A bare fused silica capillary of I.D. 50 μm was tapered and polyimide-graphite coated³⁶. The “Black Dust” electrospray emitter was inserted into a stainless steel connector that was fitted into the Analytica atmosphere-vacuum interface. Before entering the analyzer cell, ions were accumulated in a hexapole.

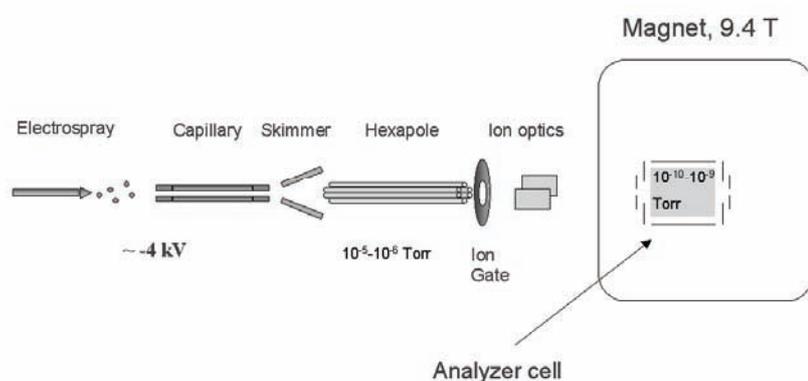


Figure 4. A cross section of the 9.4 T FTICR mass spectrometer used in the experiments described in this thesis. While ions are electrosprayed at atmospheric pressure, ultra-high vacuum is required in the analyzer cell for the generation of high resolution mass spectra.

The experiments were conducted using on-line packed capillary FTICR MS. Reversed-phase liquid chromatography (RPLC) columns were packed in-house using slurry packing. C_{18} - or C_8 -packing materials were chosen for different applications presented in the thesis. The inner diameters of the capillaries were 200 μm , and a typical column length was 10 cm. Two standard commercial LC pumps (JASCO 1580, JASCO Japan) were used to deliver mobile phase gradients of H_2O and acetonitrile. Flow rates of around 1 $\mu\text{L}/\text{min}$ were chosen over column. Before mass analysis, the analytes passed a UV-detector and the outlet of this detector was connected to the Black Dust sheathless electrospray emitter³⁶.

Mass spectra were collected on a workstation running XmassTM. The chromatography mode was selected. For LC-separations of tryptic digested proteins, 256 spectra were typically created, and each spectrum was recorded

during 10 s. The set of data collected during one experiment, providing information on m/z , signal intensity and chromatographic elution time of the analytes, is referred to as a mass chromatogram. For further analysis, including peak selection, calibration and identification, programs were written in-house. The programs constructed for different demands are described under the corresponding chapters in the thesis and in the papers.

3. Qualitative Proteomics

When investigating a complex sample, one of the first challenges might be to perform a qualitative analysis, i.e., to identify the components in the matrix. In qualitative proteomics, the goal is to identify as many proteins as possible from a cell, a body fluid or a tissue. Since thousands of proteins are present in broad concentration ranges, powerful analytical methods are needed for these studies.

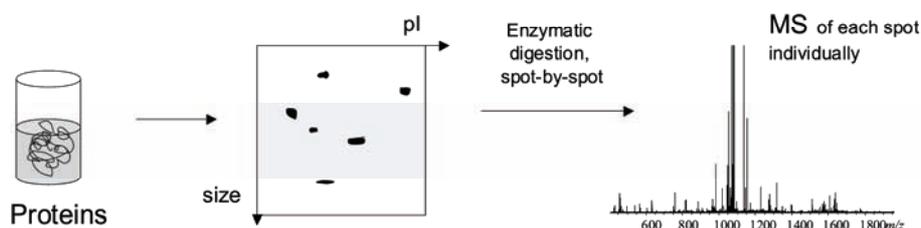
Expression Proteomics Methodology

The traditional approach to do expression proteomics involves the separation of proteins using two-dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis (2D-PAGE). This method was first described in 1975^{37, 38}. In 2D-PAGE, the proteins are separated with respect to two independent properties, isoelectric point (pI) and molecular weight, by isoelectric focusing and polyacrylamide gel electrophoresis, respectively (Figure 5a). Due to differences in pI and size the proteins appear in different areas of the gel, where they are monitored as spots. Isomers of the same proteins often appear as separate spots on the gel, since the pI is influenced by post-translational modifications, and truncations alter protein size. After separation of the proteins, the content of the spots can be examined taking different approaches. Generally, the proteins are digested by an enzyme to yield shorter peptides, after which peptide masses from each spot are detected by mass spectrometry. Each protein gives rise to a characteristic peptide pattern, and the protein can be identified by database searches against the detected peptide masses.

The standard proteomics approach is frequently applied for many purposes³⁹. However, there are also several limitations using this approach. It is time-consuming and difficult to automate. Also, it discriminates proteins of extreme hydrophobicities and sizes. Therefore, there is a need for alternative and complementary techniques. The dominating alternative approach is based on liquid separation in one or several dimensions combined with tandem mass spectrometry. Figure 5b illustrates the experimental procedure of this technique, which is often referred to as “shotgun proteomics”^{9, 40, 41}. The proteins are first enzymatically cleaved, and the resulting peptides are separated prior to MS analysis. Various combinations of liquid-based separation

methods, preferentially LC and capillary electrophoresis (CE), have been combined on-line with electrospray mass spectrometry. Physical fragmentation of the peptides provides sequence information that is utilized to identify the proteins. One of the best-known experimental procedures for shotgun proteomics is named multidimensional protein identification technology (MudPIT)^{40, 42}. If a mass spectrometer of high mass accuracy is used, the fragmentation step might be omitted and identification via peptide mapping can instead be performed⁴³.

a) Proteomics using 2D-PAGE



b) Shotgun Proteomics

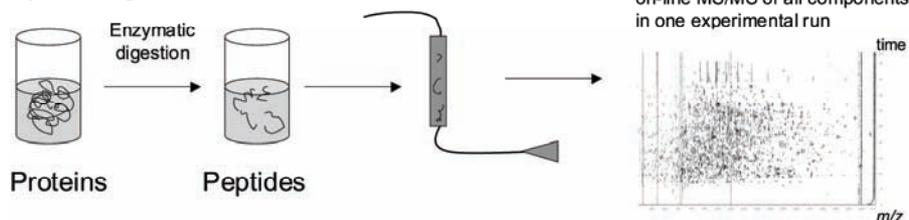


Figure 5. Description of two methods commonly applied in proteomics. a) Intact proteins are separated using two-dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis (2D-PAGE) with respect to isoelectric point and size. The protein content in each spot is then digested by an enzyme and the fragments are identified by mass spectrometry. b) In shotgun proteomics, all proteins are digested simultaneously, after which the resulting peptides are separated in one or several dimensions prior to detection by tandem-mass spectrometry.

FTICR MS in Qualitative Proteomics

Several recent review articles discuss the importance of FTICR MS in proteomics⁴⁴⁻⁴⁶. Often, the approaches are divided into two sample preparation-dependent categories: “bottom up” and “top down” proteomics. Experimental procedures involving enzymatic fragmentation of a proteome prior to

mass analysis, e.g., shotgun proteomics and peptide mass fingerprinting, are referred to as bottom up approaches. High throughput and the possibility to analyze peptides of appropriate sizes are advantageous characteristics of these methods. For example, Smith and co-workers have described an approach for bottom up proteomics based on combined high-efficient packed capillary LC and FTICR MS³²⁻³⁴, applied in the analysis of globally digested bacterial proteomes and yeast cells. The combination of FTICR MS and 80 cm long capillary columns allowed for the detection of 48,600 tryptic peptides from *Deinococcus radiodurans* cells in a single experiment. Even more impressive, 110,000 components from a yeast digest could be detected³³ when applying a multiple capillary LC-system. To accurately identify the proteins in the sample, a strategy relying on accurate mass tags was developed^{43, 47}. The accurate mass measurement information allowed the identification of ~1000 yeast proteins.

However, information on intact proteins is lost in the digestion step, and as an alternative, “top down” proteomics, the analysis of intact proteins can be applied^{44, 48}. Information on, e.g., post-translational modifications can be achieved when combining intact mass analysis with effective physical fragmentation methods. Top down proteomics generally offers a lower throughput and there is also an upper mass limit above which isotopic resolution cannot be achieved. Although Kelleher *et al.* have reported isotopic resolution of chondroitinase I, a protein with a molecular weight of 112 kDa⁴⁹, smaller proteins are more suitable for top down analysis.

Packed Capillary LC-FTICR MS of Body Fluids

The LC-FTICR MS approaches described in this thesis were developed in order to facilitate studies of very complex protein mixtures. Our main interest has been to investigate human body fluids, including plasma (**Paper IV**), cerebrospinal fluid (**Papers I, II, IV, and VI**) and amniotic fluid (**Paper III**).

Human Plasma

Human blood has long been regarded as a potential source of biomarkers. Hippocrates (400 B.C.) suggested that disease was due to imbalance of blood, phlegm, yellow bile and black bile. Today, proteomics experiments are frequently performed on plasma or serum. Plasma is the solute remaining after removal of cells from blood, while serum is blood after clotting. On average, an adult has a blood volume of 4.5 liters, corresponding to 2.5 liters of plasma.

Human plasma is one of the most complex proteomes, containing proteins acting in plasma, as well as proteins acting as messengers between tissues, and proteins leaking into the blood due to tissue damage. The total protein concentration is around 60 g/L. Twenty-two proteins constitute 99% of the total protein content⁵⁰, and the most abundant protein, human serum albumin (HSA), makes up 55%⁵¹. The dynamic range of plasma proteins covers 10 orders of magnitude!⁵¹

Cerebrospinal Fluid

Cerebrospinal fluid (CSF) is a transparent fluid that is formed mainly centrally in the brain in the *choroid plexus*. CSF circulates through the ventricles, over the surface of the brain and down the spinal chord. Its main functions are to protect the brain mechanically, and to transport metabolically active substances and waste products⁵². The fluid is secreted by active transport of solutes through epithelial cells. Water, gases and lipid-soluble compounds move freely from blood into the CSF, while macromolecules and small polar molecules do not enter through the blood-CSF barrier. Hence, the protein concentration in CSF is low, around 350 mg/L, which is approximately 200 times lower than in plasma. Most of the proteins are plasma-derived, but around 20% are produced in the central nervous system^{53, 54}. Prostaglandin H2-isomerase (β -trace protein) and cystatin C are examples of brain-derived (or enriched) proteins^{53, 54}. Other components, e.g., transthyretin, are regarded as being both blood- and brain-derived.

The rate of formation of CSF is around 0.3-0.4 mL/min, and the total volume is 100-150 mL in adults. This means that CSF is replaced 4 times every day⁵⁵. Due to the direct contact with the extracellular surface of the brain, alterations correlated with, for example, neurological disorders can be followed in CSF. The most common way to collect CSF samples for clinical examination is via lumbar puncture. Figure 6 shows the central nervous system and indicates the region where lumbar samples are drawn. From an adult, 12 mL of CSF are typically extracted. In order to avoid gradients, the sample is mixed. Cell counts are performed and then the CSF is aliquoted in portions for further analyses. It is important to choose the right sample tubes and storage conditions.

Amniotic fluid

Amniotic fluid (AF) surrounds the fetus in the amniotic cavity, protecting the fetus from mechanical and thermal shock. The total volume of AF is on average 50 mL at 12 weeks gestation, and increases with gestational age to around 1L at 38 weeks⁵⁶. Knowledge of the biochemical composition in AF is of utmost importance, since it may reflect fetus health. In early preg-

nancy, the protein levels are very low, but the protein pattern, as well as the electrolyte composition and acid-base balance, change abruptly after 10 weeks of gestation⁵⁷. The sample analyzed in **Paper III** was taken at 15 weeks gestation. At that time, fetal urine and lung fluid are the main sources of AF, while the fluid is recirculated by fetal swallowing⁵⁷. The total protein concentration is around 2.6 g/L⁵⁸, which is 20 times lower than in plasma but 7 times higher than in CSF. HSA constitute 60% of the total protein content.

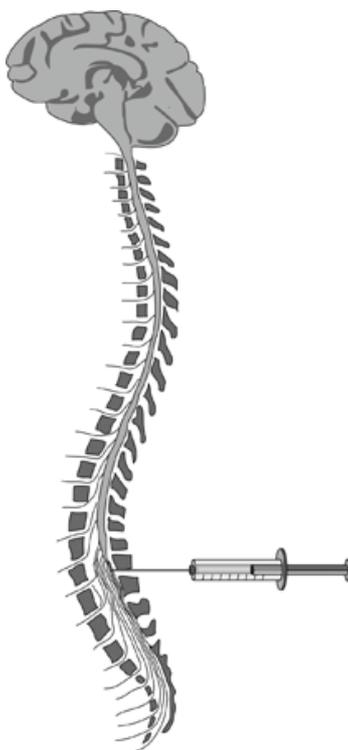


Figure 6. An illustration of CSF collection via lumbar puncture. CSF is formed mainly centrally in the brain, in the *choroid plexus*, from where it circulates through the ventricles, over the surface of the brain and down the spinal chord.

Experimental Procedure

The experimental procedures for qualitative proteomics in **Papers I, III and IV** were essentially the same. While the instrumentation was described in the previous chapter, an overview of the route for protein identification is given in Figure 7. LC-FTICR mass chromatograms of a globally tryptic digested body fluid were collected, and the neutral peptide masses were extracted. Theoretical masses of all tryptic fragments from lists of candidate proteins were calculated. In **Paper I and IV**, the list was restricted to a subproteome

of ~150 human proteins which had previously been reported in different body fluids, while **Paper III** reports on results when searching against 24,000 human entries in the Swiss-Prot/TrEMBL database. The experimental masses were then compared to the theoretical masses, tolerating a mass measurement error of a few ppm. Several matching peptides from a certain protein are required in order to give significant protein identification. The distribution of the identified peptides within the protein sequence and the mass measurement error distribution were utilized to estimate the significance of the protein identification⁵⁹. In addition, it has been shown that retention time prediction can be utilized to further exclude the risk of false matches⁶⁰. The retention time information was incorporated in the algorithm used for identification of proteins in amniotic fluid (**Paper III**).

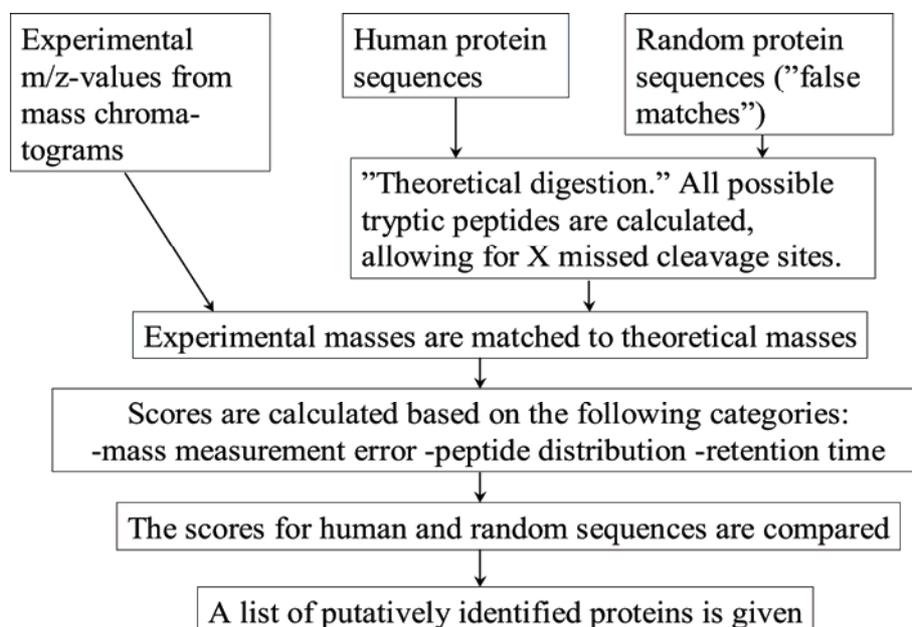


Figure 7. A description of the identification procedure used for qualitative proteomics. Measured peptide masses are compared to calculated masses of tryptic fragments from human proteins. Protein identification is based on the calculation of likelihood scores for the matching peptides.

Identification of Proteins in Cerebrospinal Fluid

Paper I describes the experiments on cerebrospinal fluid. A mass chromatogram of tryptic digested CSF contained approximately 6600 peptide masses. The procedure allowed for the identification of 39 proteins. It is interesting to note that the number of identified proteins is comparable to or

better than the results frequently achieved by the traditional 2D-gel approach⁶¹⁻⁶⁴.

In alternative studies in our laboratory, the CSF proteome was analyzed applying direct infusion⁵⁹ and on-line CE⁶⁵ FTICR MS. The three approaches are compared in Table 2 with respect to the number of identified proteins, the estimated experimental time and sample consumption. The LC-approach was proven to be the most powerful in terms of detected tryptic peptides and the number of identified proteins. As expected, both separation methods are superior to the direct infusion approach due to the reduced sample complexity in each single spectrum. The sample consumptions of all three approaches are minor, compared to ordinary 2D-gel analysis, where volumes of 100-350 μ L CSF are typically loaded^{62, 63, 66, 67}. Davidsson *et al.* have presented an alternative 2D gel approach involving prefractionation by liquid-phase isoelectric focusing^{68, 69}. Though this method has proven to allow for the detection of more protein spots than ordinary 2D-PAGE, each experimental run requires 3 mL of CSF!

It might be argued that the sample consumption is 1000 times higher in the LC-experiment than in the corresponding CE-run. However, the 32 μ L consumed in the LC experiment make up only 0.3 % of the total sample extracted by lumbar puncture, which means that the analysis can be repeated around 300 times. Also, in a proper comparison the total amount of body fluid required for sample preparation should be considered.

Table 2. A comparison of three approaches for the detection of proteins in cerebrospinal fluid by FTICR MS.

	Direct infusion	Capillary electrophoresis	Liquid chromatography
Sample consumption (μ L)	7.8	0.016	32
Experimental time (min)	40	15	60
Number of detected peptides	<1000	1500	6600
Number of identified proteins	13	30	39

The time required for one experiment is at last one of the main disadvantages of the LC-experiment. Here, a 1h-mobile phase gradient was chosen for the separation of tryptic peptides. The estimated times given in the table do not include the equilibration times required in the chromatographic runs. Even though the information from the LC- and CE-mass chromatograms is more extensive, the direct infusion approach is an attractive alternative when scanning an unknown sample due to its simplicity and ease of use. Often, an experimental time of 1-5 min is sufficient to get an overview of the components in an unknown sample. However, longer analysis times should allow

for detection of lower-abundant components and increased signal-to-noise ratios.

A General Method for Identification of Proteins in Body Fluids

The LC-FTICR MS method first described in **Paper I** is a rather general proteomics approach that can be utilized with slighter modifications to analyze other biological fluids, for example plasma (**Paper IV**) and amniotic fluid (**Paper III**). One of the limitations in the first presented reports is that ~150 human proteins were pre-selected as candidates, and thus there was no possibility to find “novel” or unexpected proteins in the fluid. The method might hence be regarded as sub-proteomic. In the study of amniotic fluid, the peptide masses were instead matched to 24,000 human proteins from the Swiss-Prot database. Peptide mapping is probabilistic and the risk when searching against this many protein entries might be that more false matches appear. To avoid this, only proteins scoring higher than the highest scoring negative control were considered as putative matches. The lists of putative proteins presented in the article include both proteins that have previously been found applying proteomics approaches based on 2D-PAGE⁷⁰ and CE⁷¹, and some novel candidate proteins. Also, the results in **Paper III** indicate the advantages of removing high-abundant proteins prior to LC-MS analysis.

Removal of High-Abundant Proteins in Body Fluids

The possibility to detect lower abundant components in body fluids is restricted, due to the presence of high-abundant proteins. The dominating protein in CSF, plasma and amniotic fluid is HSA. This protein alone makes up around 50% of the total protein concentration. Hence, removal of this and other high-concentration proteins would be beneficial.

The removal of HSA from a body fluid matrix is a challenge, and the ultimate goal is to remove this protein specifically without losing other components. There are several more or less selective methods available for this purpose. Among these, depletion columns of Cibacron Blue-Sepharose media⁷² and monoclonal antibodies⁷³⁻⁷⁵ are two dominating approaches. In case of antibody-based methods, the removal columns are often constructed also to deplete other abundant proteins, e.g., immunoglobulin G⁷³⁻⁷⁵, which further improves the chances to detect lower-abundant components.

Paper III reported that more proteins could be identified, and that the number and total intensity of peptides corresponding to other high-abundant proteins increased markedly in amniotic fluid after removal of HSA. The intention in **Paper IV** was to test and compare the performances of two commercially available HSA-removal kits when combined with the LC-FTICR MS approach. Both kits were developed for the depletion of HSA, and HSA and IgG, respectively in serum and plasma. The “HSA/IgG-

removal kit” is antibody-based, while the HSA-removal media of the “HSA-depletion kit” is proprietary. In our study, the methods were applied to plasma and CSF. To our knowledge, neither of the kits had previously been combined with CSF analysis, and hence this aspect was also of interest.

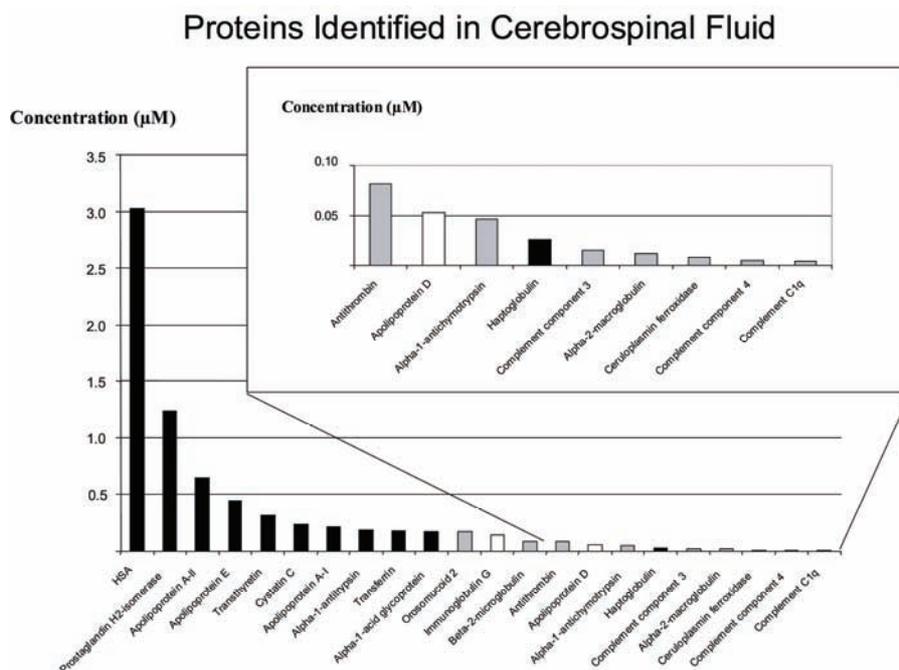


Figure 8. Proteins identified in cerebrospinal fluid applying LC-FTICR MS on native body fluid or samples treated with the HSA/IgG-removal kit. The concentrations of the detected proteins have been reported in the literature^{53, 54, 77-81}. Black color denotes proteins observed using both methods, gray and white correspond to proteins only observed after depletion of HSA and IgG, and in native CSF, respectively. The trend is that higher-abundant proteins could be observed both in native and depleted samples, while the identification of less abundant components was facilitated using the HSA/IgG-removal kit.

Most reports on depletion kits have monitored 2D-PAGE patterns prior to and after protein removal⁷³⁻⁷⁶, indicating significant reductions in spot sizes in the albumin-containing regions. The effect should be more global when performing LC-MS analyses, since peptides from the high-abundant components are spread throughout the entire separations. Increased signal intensities were also observed for tryptic peptides from other proteins than HSA and IgG after depletion. It was clear that the reduction of HSA was most pronounced using the antibody-based method, which agrees well with the instructions from the manufacturers. In order to estimate the proportion of removed HSA, a method for quantitative proteomics, described in Chapter 5,

was applied. According to our results, the two depletion methods are compatible also with CSF. However, the HSA/IgG-removal kit was proven to be more efficient and to allow for the identification of more proteins. Figure 8 compares protein identification in CSF prior to and after removal of HSA and IgG. Higher-abundant proteins were identified in both samples, while sample preparation with the HSA/IgG-removal kit allowed for the observation of lower-abundant components.

4. Comparative Proteomics

In medical research, one goal is to find biomarkers, biomolecules whose concentrations are altered in certain disorders. The identification of such components could facilitate the diagnosis of the disorder under study, improve the knowledge of its etiology, and lead to the development of new drugs. An optimal biomarker should be precise and disorder-specific, and also it should be technically easy to observe.

Methods for Comparative Proteomics

Expression proteomics opens up the possibility to compare the proteomes, or subproteomes, of, e.g., body fluids of patients and healthy controls. Traditionally, methods for the quantification of pre-selected proteins, one-at-a-time, have been applied to reveal biomarkers⁸². Immunoassays, including enzyme-linked immunoassays (ELISA) and radioimmunoassays (RIA), are the workhorses in these studies^{78-80, 83, 84}. In contrast, proteomics permits a faster and more discovery-oriented approach to the problem. When constructing a method for biomarker discovery, it is important to take into account that protein and peptide concentrations vary naturally between individuals. Hence, ranges or threshold-values need to be defined to distinguish natural biological variations from disease-correlated variations.

Even though 2D-PAGE is not strictly quantitative, alterations in the protein levels are often readily detectable when comparing series of samples⁸⁵. Both 2D-PAGE and LC-MS experiments of proteomes give rise to very complex protein or peptide patterns, and so advanced and suitable data handling is required before any conclusions can be drawn. There are today powerful commercially available pattern recognition programs for image analysis of 2D-gels. However, the identification of matching spots might be difficult due to the variability between gels, and data evaluation often require an experienced operator^{85, 86}. Comparative analyses of LC-MS data are as complex as the analysis of gels, but today not as widespread. Pattern recognition algorithms, developed for the comparison of mass chromatograms, need to compensate for between-sample variations in chromatographic elution times and smaller mass shifts. Fluctuations in signal intensities and noise levels should also be considered^{87, 88}.

Development of a Method for Comparative Proteomics using LC-FTICR MS

Knowing that LC-FTICR mass chromatograms of body fluids were informative and extensive, the approach was further developed for comparative proteomics (**Paper II**). A method suitable for the analysis of CSF is of interest in research concerning, e.g., neurodegenerative disorders. In collaboration with the Unit of Neurology at the University hospital, a project aiming at comparing the protein repertoire of CSF from patients suffering from amyotrophic lateral sclerosis (ALS) and healthy controls were initiated. Mass chromatograms of CSF from ALS-patients and healthy controls were generated according to the experimental procedure described in **Paper I**, and the protein patterns achieved from the two sample categories were compared.

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis is a neurodegenerative disease, characterized by the degeneration of motor neurons in the motor cortex, brainstem and spinal chord. Approximately 2-4 of 100,000 persons suffers from the disease. In Sweden, there are around 170 new cases every year. The disease is relentlessly progressive and the patients die from respiratory failure within 3-5 years after onset.⁸⁹ The primarily underlying mechanisms for the disorder are today unknown.

Most ALS cases are sporadic, but 10% of the cases are familial⁹⁰. It has been found that 2% of all ALS and 20% of the familial cases are correlated with a mutation in the gene coding for copper/zinc superoxide dismutase, SOD1⁹⁰⁻⁹². Protein accumulations have been associated with several neurodegenerative disorders, including Parkinson's disease, Alzheimer's disease and ALS⁹³⁻⁹⁵. Neurofilament mutations⁹⁵ and elevated levels of neurofilament triplet protein (NFL) in CSF⁸⁴ have been correlated with ALS. However, these findings are not unique to the disease and have been observed also in other neurological disorders.

Description of the Pattern Recognition Program

In order to compare the mass chromatograms, a pattern recognition program was constructed in-house. The main demands on the program were that it should extract classification features from two different groups, and then be able to classify unknown samples into the correct group. In a mass chromatogram, the m/z -value, the elution time and the intensity of each peak are given. However, due to instrumental drift and aging of the column, these parameters might vary with time. The first step of the pattern recognition algorithm did hence deal with calibrating the mass chromatograms in time

with respect to a “standard mass chromatogram”. High-abundant signals common to all pictures were selected and a polynomial function was adjusted to fit the time profiles. After time calibration, the time dispersion between samples was less than 2 spectra (20 s). Before continuing the analysis, the signals were also normalized to the average signal intensity.

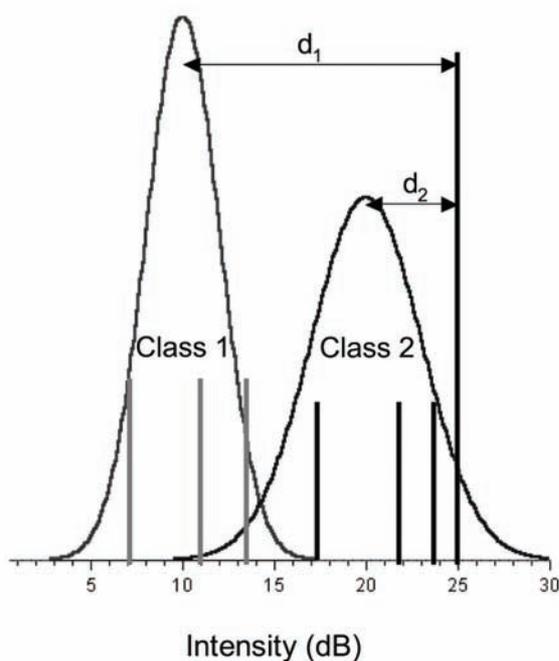


Figure 9. An illustration of signal intensity distributions for one classification peak. Gray lines denotes signal intensities detected in mass chromatograms from Class 1 (e.g., controls) and black lines those from Class 2 (for example patients). Average intensities and standard deviations of the distributions were calculated. The long black bar denotes the intensity of an unknown samples. By calculating normalized distances to both classes, for each classification peak, the probabilities of belonging to one class or the other were estimated.

The next step was to extract classification features of the two classes. Intensities of all peaks in the mass chromatograms were extracted and compared. A number of samples from each group were used as classification samples. Gaussian distribution was used to describe the dispersion of peak intensities in a given peak position of the mass chromatograms (Figure 9). If the intensity distributions of the two classes were clearly separated, the peak was selected as a classification peak.

To be able to classify unknown samples, an estimation of the probability of belonging to either of the two classes is needed. This was achieved by calculating so called Mahalanobis distances. For each classification feature, the intensity of the corresponding peak was extracted and normalized distances were calculated according to

$$D_i = \frac{d_i}{\sigma_i} \quad (5)$$

where d and σ are the distance to the average intensity of a classification peak, and the standard deviation of the peak intensities within a class, respectively (see Figure 9). The average of the D -values from all classification peaks in one sample was then calculated to estimate the Mahalanobis distance $\langle D \rangle$ and the probability, P , of this sample to belong to one class or the other. The probability is proportional to $\exp(-\langle D^2 \rangle)$.

Evaluation of the Results

The first question to be addressed was whether finding a potential biomarker was at all possible in the complex mass chromatograms, given a limited number of samples. To test this, CSF from healthy donors were spiked with an *in vitro* added biomarker, tryptic digested myoglobin from horse, in two different concentrations, 1.4 and 0.14 μM . Mass chromatograms of these samples and of native CSF were collected. Even though a large variation in the number of detected peptides between samples was observed, peptides from the *in vitro* added protein were extracted as classification features. Also, a 10-time up-regulation of this component could be detected.

In order to search for potential biomarkers of ALS, mass chromatograms were collected for 12 patients and 10 controls. When applying the algorithm to the ALS- and control samples, the peptide patterns were clearly separated, and four out of five test samples were classified as belonging to the correct group (Figure 10). However, the peptides that were found to differ in intensity could not be traced back to one specific protein. Most probably, the peptides giving rise to the classification pattern originate from more than one protein. This complicates the database searches, using the available search algorithms. It should be valuable to run tandem mass spectrometry of the classification peptides to confirm their identities.

The results are interesting from several points of views. The methodology is novel for this type of applications. Today, the diagnosis of ALS is based on clinical and neurophysiological examination⁹⁶. Even though the origins of the classification peptides are not known, it is possible that the MS-approach, after further development, might be used as a novel tool in diagnosis of ALS. The results from the myoglobin-spiked samples indicated that

a number of possible “false” classification peptides should be expected, since only a few of the classification peaks originated from myoglobin. This is probably due to both biological and experimental variations. It will be interesting to analyze more samples to improve the statistics and exclude coincidental matches. As expected, the between-day variation of the experiment exceeded the within-day variation, why analysis during shorter periods of time should be preferred.

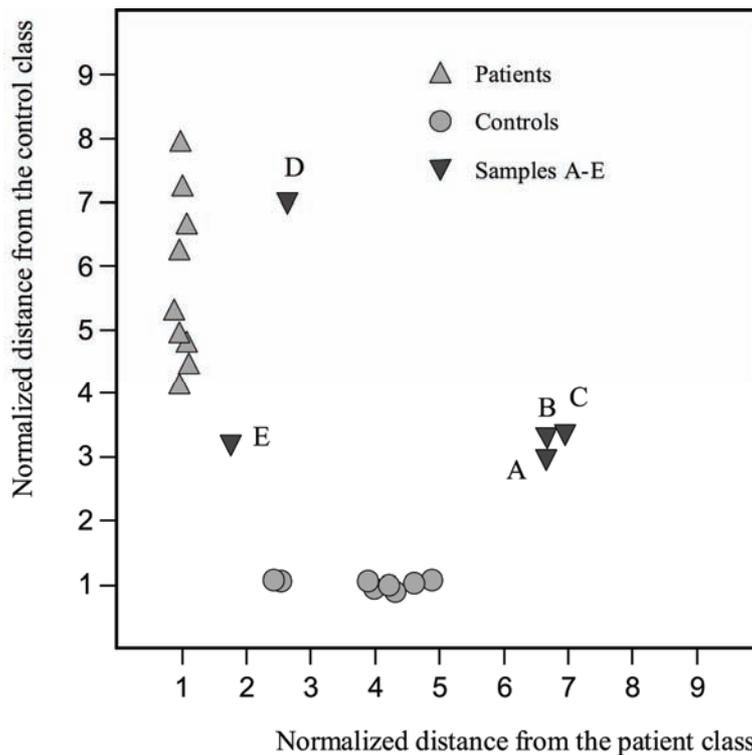


Figure 10. A Cooman plot demonstrating the classification result. The patient and control samples used for classification are well separated. The “unknown” samples (A-E) should be classified into the group towards which the normalized distance is the shortest possible. Hence, samples D-E should originate from ALS-patients, and samples A-C from controls. The results are correct, except for patient B who suffers from ALS.

There are today no known disease-specific biomarkers for ALS. To rule out whether the characteristic peaks found in the present study are unique to ALS, CSF from patients suffering from other neurodegenerative disorders, e.g., Parkinson’s disease and Alzheimer’s disease, will be analyzed.

5. Quantitative Proteomics using Chemical Labeling

Direct comparison of 2D-PAGE patterns or mass chromatograms allows for observations of up- and down-regulations of protein expression. However, more precise quantitative information is not possible to achieve. Experimental variations complicate the image analysis, and in many cases, the patterns are not perfectly superimposable even after proper calibration. Alternative strategies permitting relative quantification of proteins and peptides in two samples, or pools of samples, rely on chemical labeling.

Labeling Techniques

Several labeling techniques have been developed for mass spectrometry-based analysis^{32, 85, 97-100}. In a typical experimental procedure (Figure 11), two samples, representing different conditions, are tagged with either of two markers of similar chemical properties, differing slightly in mass. Generally, the markers differ by stable isotope substitution at some given positions. After labeling, the two sample pools are mixed and analyzed in the same MS-event. Peptide pairs of characteristic mass difference are identified in the mass spectra. The relative intensity ratios correspond to the relative expression of the proteins or peptides in the original samples. Similar approaches have been applied to overcome problems with between-gel variability. In difference gel electrophoresis, two samples to be compared are labeled with either of two different fluorescent dyes prior to 2D-PAGE. A third dye is also available, which makes it possible to include an internal standard in the analysis. The images are visualized using fluorescent scanning at separate wavelengths^{39, 101, 102}.

Isotopic labeling of peptides prior to mass spectrometric detection has been performed both *in vivo* and *in vitro*. *In vivo* experiments are appropriate for determining differential protein expression in cell cultures, grown under different conditions. The approach is also referred to as metabolic labeling. For example, LC-FTICR MS has been applied for quantitative analysis of bacterial and mammalian cells that were cultured on normal and ¹⁵N-enriched media^{32, 100}. However, metabolic labeling cannot be combined with

human samples, such as body fluids. The number of incorporated stable isotopes is also dependent on peptide composition, and hence it is difficult to predict the mass difference between non-labeled and isotopically labeled peptides.

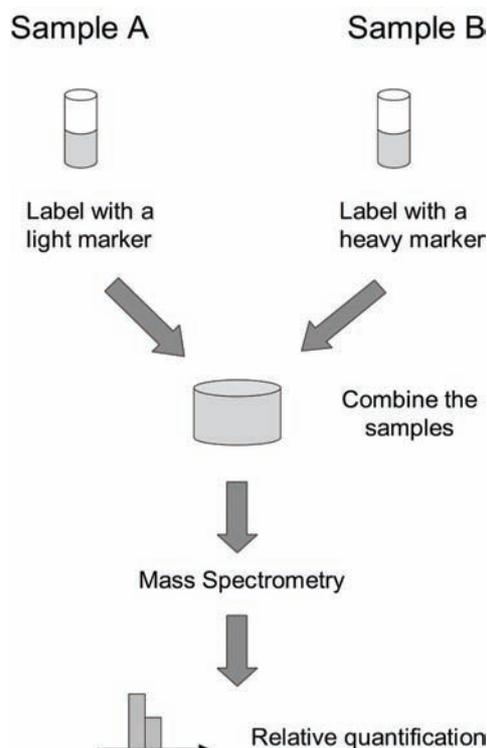


Figure 11. Quantitative proteomics using chemical labels. Peptides in two samples are tagged with either of two chemical markers differing slightly in mass. After labeling, the samples are mixed and analyzed in the same MS-event. The relative intensities of light and heavy labeled peptides in the mass spectrum correspond to the relative amount of these components in the original samples.

Several different sets of chemical markers have been developed for *in vitro* labeling. Labeling can be either global, i.e., directed towards a chemical group present in all peptides, or amino acid-specific. The best-known technique for relative quantification is the isotope-coded affinity tags (ICAT) strategy⁹⁸. In this approach, the cysteine residues are alkylated with either a light or a heavy reagent. The reagents consist of three different parts: an *affinity tag*, which is used for isolation of the labeled peptides, a *linker* and a *reactive group* with specificity towards the thiol group. The two labels are identical except for at eight hydrogen positions in the linker, where normal hydrogen is substituted for deuterium in the heavier variants. Thus, the mass difference induced by tagging a peptide containing one cysteine residue is

eight Da. After labeling and enzymatic cleavage, the tagged peptides are isolated for further analyses. This step results in a significant reduction of sample complexity, since only a limited number of peptides are chosen for mass spectrometric analysis. Variants of ICAT that differ from the initial approach both in the sample preparation steps¹⁰³ and in the choice of stable isotopes¹⁰⁴ have been reported. Another amino acid selective method is called mass-coded abundance tagging (MCAT)⁹⁹. MCAT involves the guanidination of lysine residues.

A limitation of amino acid-directed markers is that valuable information is lost in the pre-selection step. It has been calculated that 11% of all human proteins and 83% of the tryptic peptides produced from human proteins lack cysteine residues⁹⁷, and hence ICAT is not suitable for the analysis of these components. In global labeling techniques, peptide N- or C-termini may be targets for the reagents. One strategy involves the incorporation of ¹⁸O atoms into the carboxyl termini of tryptic peptides^{105, 106} during the digestion step when one pool is incubated in ¹⁸O-enriched water. N-terminal labeling has been achieved by acetylation¹⁰⁷ and nicotinylation¹⁰⁸ of the amino group with isotopically (H₄/D₄)-labeled reagents.

Quantification Using Enhanced Signal Tags

Our group has combined an alternative method for relative quantification with electrospray FTICR MS, namely the quantification using enhanced signal tags (QUEST) method¹⁰⁹ (**Papers IV, V and VI**). This is a global labeling technique in that it involves labeling of the N-termini of all peptides. In addition, lysine residues are amidinated by the markers. The two reagents, S-methyl thioacetimidate (SMTA)¹¹⁰ and S-methyl thiopropionimidate (SMTP)¹¹¹ (Figure 12), were synthesized in-house. The chemical difference between the two reagents is a methylene group, which results in a mass difference of 14 Da.

Initially, the method was developed for MALDI-experiments¹⁰⁹. The first study focusing on QUEST-markers in combination with ESI-MS is presented in **Paper V**. Two pools of the same tryptic digest of bovine serum albumin (BSA) were labeled with the light and heavy marker, respectively. Samples containing different concentration ratios of the two pools were prepared and analyzed by direct infusion ESI-FTICR MS. The intensity ratios of all detected peptide pairs were calculated and the median values were considered as the best estimation of the relative concentration. However, the standard deviation for each sample was huge. This is due to sample complexity and the risk of assigning “false” pairs, rather than incomplete labeling of the sample peptides.

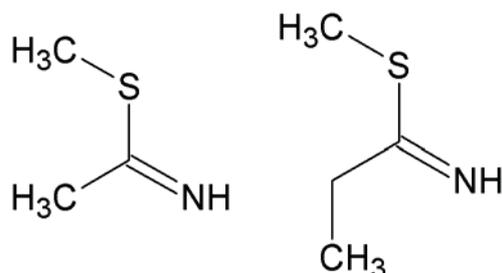


Figure 12. The two amidination reagents, S-methyl thioacetimidate (SMTA), to the left, and S-methyl thiopropionimidate (SMTP), to the right, used as QUEST-markers. The difference between the molecules is a methylene group, and hence the corresponding mass shift is 14 Da.

In order to investigate more complex samples and to be able to detect larger concentration variations, the labeled peptides were separated on packed capillary LC prior to FTICR MS. Since the two QUEST-markers differ by a methylene group, a peptide labeled with the SMTP-reagent will be more hydrophobic than the same peptide labeled with SMTA, and a slight difference in elution time should be expected. On the contrary, stable isotope-labeling results in coelution¹⁰⁴ or nearly coelution⁹⁸ of labeled pairs, which should reduce the risks of variations due to electrospray fluctuations and suppression effects. In the study, a C₈-packing material was chosen for RPLC. Under the given conditions, a typical difference in elution time between SMTA- and SMTP-labeled peptides was 70 s, corresponding to 7 mass spectra. Despite this, the obtained results for the lower concentration ratios 1:1 and 2:1 were consistent with the expected values (Table 3). The results were well comparable to those achieved when applying stable isotope labeling techniques, such as ICAT⁹⁸ and ¹³C-ICAT¹⁰⁴. The last value in Table 3 indicates a 7-time concentration difference between the samples, while a 5-fold alteration was prepared. However, previous studies on QUEST-markers¹⁰⁹ and ICAT⁹⁸ have also reported larger divergences from the true values if higher concentration ratios are to be determined. Even though the result is not exact, it is clear that a significant alteration has occurred. To achieve more precise values for larger concentration differences, dilution or up-concentration of either of the investigated samples is recommended. Also, a more selective choice of peaks for the determination of median intensity ratios should narrow the observed range by excluding false matches.

Table 3. Experimental and theoretical concentration ratios of QUEST-labeled tryptic digests of BSA. The experimental median values of the lower concentration ratios agree well with the known values.

Prepared concentration ratio	Number of assigned matches	Measured intensity ratio	
		Median	Range
1.0	84	0.95	(0.05-16)
2.0	70	2.14	(0.16-21)
5.0	40	7.18	(1.8-170)

QUEST-markers for the Quantification of Proteins in Body Fluids

The goal of using quantitative proteomics is to find relevant up- or down-regulations of proteins in biological matrices, e.g., body fluids, due to certain conditions. The use of global labeling techniques leads to very complex peptide patterns. A study was conducted in order to investigate the performance when QUEST-labeling tryptic digested CSF (**Paper VI**). Samples were prepared to allow for studies of the influence of both the tryptic and the labeling events. In addition, another pair of quantification markers, 1-([H₄]-nicotinoyloxy) and 1-([D₄]-nicotinoyloxy) succinimide esters¹⁰⁸, was applied for similar analysis. This pair has the advantage that two stable isotopes are incorporated, and therefore the difference in elution times on RPLC was minor. The obtained median intensity ratios for HSA and transferrin, two highly abundant proteins in CSF, corresponded well to the expected concentration ratios. From the results it can be concluded that up- or down-regulations larger than 30 % should be possible to detect using either of the methods. Despite the fact that the QUEST-labeled peptides eluted at different times, the results were of high quality also when analyzing a complex protein digest. In fact, the concentration ratios obtained with the QUEST-marker methodology showed less variation than those obtained with the [H₄]/[D₄]-labels.

As described in Chapter 3, two depletion kits for removal of high-abundant proteins were tested in **Paper IV**. The QUEST-marker methodology was applied to estimate the success rate of the removal of HSA. Our result showed that 77% and 99% of the initial HSA was removed, applying the HSA-depletion and the HSA/IgG-removal kits on plasma, respectively. These values were in accordance with what was stated by the manufacturer. The corresponding values for CSF were 51 and 98%. Hence, both kits al-

lowed for the removal of HSA also from CSF. However, it can be concluded that the combination with the HSA/IgG-removal kit was the most efficient approach. There are today several antibody-based depletion kits available, which remove more components than HSA and IgG. Present work at the department involves the integration of a 12-component removal kit (Genway Biotech, Inc.) with the LC-FTICR MS approach. However, there is also a risk associated with removing high-abundant proteins from the sample. HSA is a carrier molecule and it cannot be excluded that fractions of some less abundant components are removed, even though our results and those of other groups indicate enhanced detection of many proteins.

6. On-line Electron Capture Dissociation and LC-FTICR MS

As described in the previous sections, soft ionization techniques in combination with FTICR MS allow for proper mass determination of protonated peptides. However, in many cases additional information on peptide sequence is needed, e.g., for the identification of peptides, the localization of post-translational modification or in conformational studies. One solution is to apply tandem mass spectrometry, MS/MS. MS/MS involves the selection and activation of a known precursor ion followed by mass analysis of its fragments¹¹². Several ion fragmentation techniques have been developed for mass spectrometric applications and are today regarded as standard methods. For example, shotgun proteomics and various top down approaches rely on tandem mass spectrometry. This chapter will describe the integration of a rather novel fragmentation technique, electron capture dissociation (ECD), with on-line LC-FTICR MS.

Peptide Fragmentation

Peptide cleavage may result in different types of fragments. The standard nomenclature for peptide fragmentation¹¹³ is illustrated in Figure 13. N-terminal fragments are referred to as *a*-, *b*-, *c*-ions, while *x*-, *y*- or *z*-ions contain the C-terminal of the intact peptide. The choice of fragmentation technique will influence what types of ions to expect after fragmentation. ECD and collision-induced dissociation (CID) have been integrated in the experiments described in **Papers VII** and **VIII**. Other examples of fragmentation techniques used in FTICR MS are infrared multi-photon dissociation^{31, 114, 115} and blackbody infrared radiative dissociation¹¹⁶.

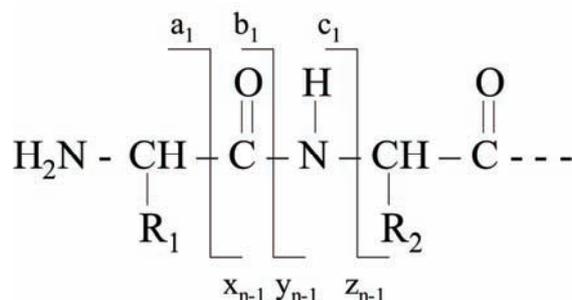


Figure 13. The commonly used nomenclature for peptide fragmentation¹¹³. Depending on cleavage site, N-terminal fragments are denoted *a*-, *b*- or *c*-ions, while C-terminal fragments are referred to as *x*-, *y*- or *z*-ions.

Collision-Induced Dissociation

The most commonly used ion activation procedure is collision-induced dissociation. In this approach, the precursor ions are accelerated to collide with neutral gas molecules. Some of the translational energy is then converted to internal energy, which induces fragmentation. Collision-induced dissociation results in formation of *b*- and *y*-ions, but *a*-ions can also be observed. There are many variants of CID, obtained in different parts of the mass spectrometer. In FTICR MS, sustained off-resonance irradiation (SORI) is provided in the analyzer cell. Briefly, a low-amplitude rf-excitation pulse is applied, a few hertz off-resonance from the cyclotron frequency of the precursor ion. The excitation frequency is alternately in phase and out-of-phase with the ion, and thus its trajectory will alternately shrink and expand²². The pressure is raised in the cell by admitting a collision gas through a pulsed valve. Multipole storage-assisted dissociation (MSAD)¹¹⁷ and nozzle-skimmer fragmentation are examples of CID-events occurring outside the cell, in higher pressure-regions of the FTICR. Consequently, precursor ion selection is not possible using the instrument applied for the experiments in this thesis. MSAD is induced through extended accumulation of ions in an RF multipole (in our case the hexapole). Nozzle-skimmer fragmentation is obtained by increasing the potential between the nozzle and the skimmer. In this way the ions are accelerated into rest-gas molecules, which will induce fragmentation.

Of great interest in this thesis is the integration of MS/MS-techniques with on-line liquid separation FTICR MS. Li *et al.* have demonstrated the combination of multiplexed SORI with LC FTICR MS¹¹⁸. The method was applied on a standard protein digest of BSA and a complex whole cell digest from *Deinococcus radiodurans*. A combination of MSAD and nozzle-

skimmer fragmentation was integrated with LC-FTICR MS for the analyses of human liver diacetyl reducing enzymes¹¹⁹. Fragmentation was generated in alternating spectra, to yield information on parent ions and fragments. Sequence-tag information was generated for proteins on a subpicomole level.

Electron Capture Dissociation

Electron capture dissociation is a rather novel fragmentation technique, first presented in 1998¹²⁰. This method is in many ways complementary to traditional fragmentation methods, including CID. Among the advantages of ECD in proteomics are the possibilities to preserve labile posttranslational modification and to provide cleavage of disulfide bonds. ECD is today an established technique only in FTICR MS. The reasons for this are mainly that the total time and low electron energies required for high fragmentation efficiency are difficult to achieve in other types of mass spectrometers¹²¹. However, a few groups have recently shown the possibility to implement the method in ion traps^{122, 123}.

Electron capture dissociation results from the interaction of multiply charged polypeptides with low-energy electrons (preferentially <0.2 eV) within the analyzer cell. The mechanism of ECD is still debated and poorly understood. Briefly, electron capture is followed by charge neutralization, radical formation and subsequent peptide fragmentation. The resulting peptide fragments are mainly *c*- and *z*-ions (Figure 13). Technically, the electrons are emitted in short pulses from the electron gun. In the initial experiments, directly heated cathodes¹²⁴ were used as electron sources, but indirectly heated dispenser cathodes have shown advantageous properties¹²⁵, and are currently the electron guns of choice.

ECD Combined with On-line LC-FTICR MS

The implementation of indirectly heated dispenser cathodes greatly reduced the irradiation times required to generate ECD conditions, from typically 3-10 s¹²⁴ to 10-100 ms¹²⁵. Hence, it is now possible to integrate this fragmentation technique with on-line liquid separation techniques. The first reports on on-line LC-ECD-FTICR MS were published in 2002 by our group (**Paper VII**) and by Davidson and Frego¹²⁶. The studies were performed on standard peptides and enzymatic digests of standard proteins. Later on, the possibility to integrate a faster separation method, CE, on-line with ECD was also demonstrated¹²⁷.

Paper VII describes ECD-fragmentation of a standard peptide mixture and a tryptic digest of BSA. No precursor ion selection was performed, and

ECD conditions were created in every other spectrum. The signals appearing in alternating spectra were considered as possible fragments. Successful fragmentation was observed for three of the standard peptides and many of the BSA-fragments. With regard to the previously described papers included in this thesis, the results obtained for enzymatic digests should be of significant interest. **Paper VII** reports that the sequence-tag information achieved from any of 13 BSA peptides was extensive enough to correctly identify the protein in a Mascot¹²⁸ search. However, a limitation when fragmenting tryptic peptides by ECD is that the precursor ions must be multiply charged. Generally, the peptide masses of the components in a near-complete tryptic digest are in the range of 800-4000 Da. The lower-mass peptides are often singly charged, and consequently ECD results in uncharged molecules that cannot be detected by MS! This reduces the possibility to use the suggested approach to investigate all parts of the protein. On the other hand, if the aim of the study is to identify the proteins in, e.g., a complex mixture, information on one single peptide might be sufficient.

The Method Applied to Peptides in a Tissue Extract

The first application of the described experimental approach to a biological sample is reported in **Paper VIII**. The sample of choice was a peptide extract from mouse pancreatic islets. Pancreatic islets are built up of different types of endocrine cells, including glucagon-producing α -cells and insulin-producing β -cells. Insulin was one of the first peptide hormones to be discovered^{129, 130}, and due to its importance in regulating carbohydrate and fat metabolism, endocrine peptides from pancreatic cells have been thoroughly investigated. There is no defined mass cut-off between proteins and peptides, but peptide masses are often below 10 kDa. Hence, traditional 2D-gel separations of these molecules are not possible. Instead, the classical method for peptide analysis relies on purification in a series of liquid chromatography steps^{131, 132}, and subsequent characterization by Edman degradation or MS. This is a rather time-consuming approach that requires a lot of material. Recent development in both chromatography and MS has by far increased the speed and sensitivity of peptide analyses.

In the approach described in **Paper VIII**, two different fragmentation methods, nozzle-skimmer fragmentation and ECD, were combined with on-line LC FTICR MS in order both to compare the performances of the different techniques, and to achieve more information on the peptides under study.

Table 4. A comparison of the number of fragments generated by nozzle-skimmer fragmentation and electron capture dissociation in studies of endocrine peptides from mouse pancreatic islets.

Name	Detected fragment ions	
	LC-nozzle-skimmer FTICR MS	LC-ECD FTICR MS
Insulin 1	Loss of water	c_3-c_5, z_6-z_7 from the B-chain
Insulin 2	Loss of water	c_3-c_5 from the B-chain
C-peptide 1	$b_5-b_{13}, b_{15}, y_5-y_{14}$	-
C-peptide 2	$b_7-b_{15}, b_{18}, y_6-y_7, y_9-y_{10}, y_{13}, y_{16}-y_{17}$	c_9-c_{12}
<i>Des</i> -(25-29)-C-peptide 1	$b_{10}-b_{11}$	-
<i>Des</i> -(27-31)-C-peptide 2	$b_6-b_{15}, b_{23}-b_{25}, y_{11}-y_{15}, y_{17}, y_{19}$	-
Glucagon	-	-
Glicentin-related pancreatic peptide	$b_{21}-b_{29}, y_{19}, y_{21}, y_{24}, y_{25}$	$c_5-c_8, c_{11}-c_{12}$

Indeed, the two methods yielded complementary information. Table 4 shows some of the detected pancreatic peptides and the number of observed peptide fragments using the different experimental routes. Generally, the nozzle-skimmer fragmentation approach gave rise to more intense fragments and more extensive sequence information. On the other hand, the ECD approach provided sequence-tag information on the two variants of insulin. The latter finding is supported by the facts that ECD has proved to be suitable for the fragmentation of disulfide-containing peptides^{133, 134}, while CID of these components is less efficient^{134, 135}. One of the drawbacks of ECD is its relatively low efficiency, which is typically 20-50% for peptides¹²¹ even under optimized conditions.

Initial direct infusion experiments on the pancreatic peptide extract showed, as expected, the presence of peptides derived from proinsulin. An interesting finding was that the detected masses of two intense signals agreed on the ppm-level with masses corresponding to a truncated form of the insulin C-peptides, lacking the five C-terminal residues. The sequence information achieved in the LC-nozzle-skimmer fragmentation experiments further confirmed the identities of these peptides (Figure 14 and Table 4).

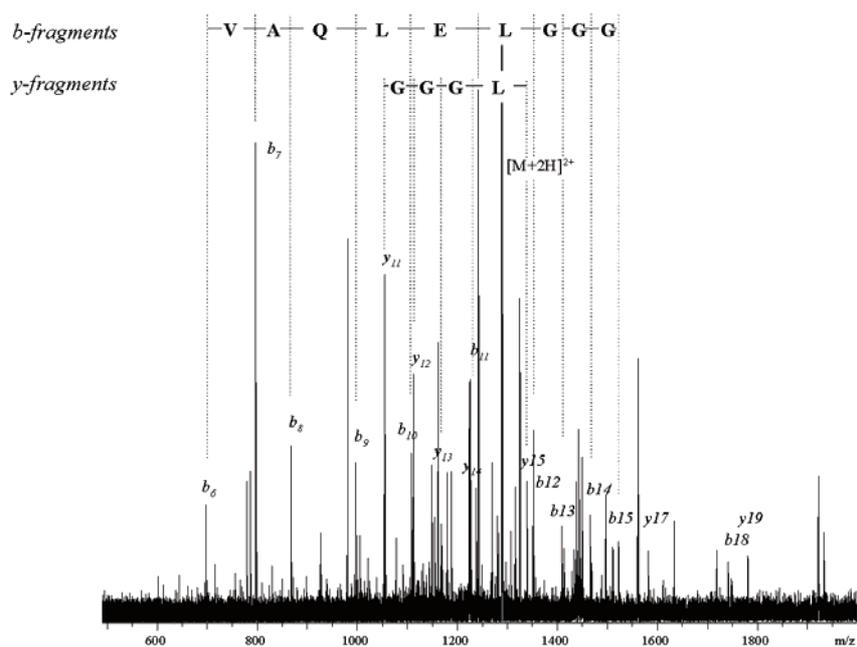


Figure 14. Sequence-tag information on the *des*-(27-31)-C-peptide 2, generated by on-line nozzle-skimmer fragmentation LC-FTICR MS. Consecutive series of *b*- and *y*-ions allowed for identification of this peptide.

Previously, the truncated form has been identified as a product from rat pancreatic islets¹³⁶. The sequence of C-peptide 1 from mouse differs from the corresponding peptide from rat, while C-peptide 2 is the same in both species. The C-terminal pentapeptide of the C-peptide is believed to be critical for bioactivity, and hence the function of the truncated form should differ from that of the intact peptide. It is interesting to note that the truncated C-peptide has been reported not to be a major product from human pancreas¹³⁷.

Of much interest would be to integrate the on-line ECD approach in the analysis of body fluids, which should facilitate the identification of unknown peptides. The approach was tested on a tryptic digest of CSF (unpublished data). However, sequence information was only achieved for the abundant HSA-fragments. It was concluded that before the method can be integrated into more complex sample analysis a higher ECD-efficiency is required. In addition, it would be advantageous to use more efficient liquid separation methods and to involve data-dependent precursor ion selection in the experimental procedure.

7. Conclusions and Future Aspects

Mass spectrometry is today one of the most powerful tools in protein and peptide research. Fourier transform ion cyclotron resonance mass spectrometry possesses several extraordinary properties, i.e., ultra-high mass resolution, mass accuracy on the ppm-level and high sensitivity. This thesis describes and discusses novel methods for the analysis of protein expression in human body fluids and peptide identification in tissue extracts, based on on-line packed capillary liquid chromatography FTICR MS.

A qualitative proteomics method, relying on high mass measurement accuracy, was initially developed for human CSF. Thirty-nine proteins could be identified from 6600 tryptic peptides. This is well comparable to the number of observed proteins in more time- and sample- consuming standard proteomics experiments. The LC-FTICR MS approach was proven to be compatible also with human plasma and amniotic fluid, after only smaller modification of the initial protocol. To further improve the sensitivity of this method, more efficient liquid based separations should be included. Such systems may involve one or several orthogonal chromatographic steps. Extra attention should also be paid to mass calibration. An even higher mass measurement accuracy would minimize the occurrence of false protein matches⁴⁷.

A comparative proteomics study was conducted in order to, if possible, reveal protein patterns correlated with a neurodegenerative disorder, amyotrophic lateral sclerosis. Characteristic tryptic peptides in CSF of ALS-patients were identified, but these could not be traced back to a specific protein. However, four out of five unknown samples were correctly classified by this approach. Future work involves the analysis of more patient and control samples, and classification of samples from patients suffering from related disorders, in order to rule out whether the findings are specific for ALS. If protein profiling of CSF by LC-MS can be used as a diagnostic tool, this is indeed a novel and tempting strategy! As an alternative, chemical labeling can be involved to compare two pools of samples, e.g., patients and controls. Global labeling by QUEST-markers was proven to be as valuable quantitative technique when combined with LC-FTICR MS. Future studies in our laboratory include QUEST-labeling of CSF-pools from the patients and controls analyzed in the comparative study. It will be interesting to compare the results of the two methods. However, it is likely that the increased sample complexity induced by global peptide labeling will limit the possi-

bilities to observe lower abundant classification peptides. As described in the thesis, one method to reduce the dynamic range of proteins in the samples is to remove highly abundant components. There are many methods and kits available on the market for this purpose. An antibody-based method was demonstrated to be highly efficient for removal of HSA and IgG both in plasma and CSF. Before including such a sample preparation step in comparative or quantitative proteomics it is important to estimate the reproducibility of the method. The depletion methods tested in these experiments were developed for serum and plasma. A trend is to construct depletion strategies permitting the removal of several abundant proteins in the same step. The relative protein abundance in plasma differs from that in CSF, and so body fluid-specific methods should ideally be developed.

Tandem mass spectrometry is commonly applied for the identification of peptides in unknown samples. So-called shotgun proteomics is based on liquid separations combined with MS/MS. The combination of ECD with LC-FTICR MS has the potential of becoming a powerful tool for peptide analysis, since ECD is a mild and complementary fragmentation technique that allows for identification and localization of labile post-translational modifications. This strategy was applied on an extract of peptides from mouse pancreatic islets. However, the efficiency of the fragmentation technique needs to be improved before the LC-FTICR MS approach will be functional in complex sample analysis.

Today, it is possible to create huge amounts of mass spectrometric data in short periods of time. The limiting step is often data-evaluation. In the future, existing procedures need to be automated, while novel proteomics approaches require the construction of fast and intelligent new programs. A close collaboration between scientists in the fields of mass spectrometry, statistics and computer science is suggested in order to meet the demands. Mass spectra and mass chromatograms are, in addition, presented in various formats, which complicates comparisons between instruments. A standardized representation of MS-data has recently been suggested¹³⁸. If this could be realized, LC-MS would be an even more competitive alternative to traditional proteomics!

8. Acknowledgements

Throughout the years as a PhD-student, I have had the benefit to join an interdisciplinary team of skilful researchers. This thesis would never have been completed without the contributions from many persons.

First of all, I would like to thank my three supervisors for all support during these years. From start, *Jonas Bergquist* had a thousand intelligent ideas about the project and showed never-ending enthusiasm about neurochemistry and mass spectrometry. I am glad that we could realize some of your many ideas and find new solutions to various kinds of problems. *Per Håkansson* encouraged me to continue in the field of mass spectrometry already during my degree project. I am most grateful for every-day discussions and your professional guidance in the FTICR-laboratory. The position was designed for me by *Karin Markides*, who very much believed in this project. I am very happy that I could join your team and learn more about analytical chemistry!

The years as a PhD-student would never have been the same without my office-mate *Charlotte Hagman*. Thank you for all interesting, fruitful scientific collaborations and discussions, shared laughs, for being a reliable travel companion and a good friend in private life!

As the project has evolved, *Johan Kjellberg* has come up with clever technical solutions, and I am convinced that my work would have taken twice as long time without your assistance! *Igor Ivonin* did a great job in constructing the pattern recognition program for mass chromatograms. You were the first person who really appreciated the huge data files I created in the laboratory.

My project has involved many interesting collaborations. I would like to thank *all coauthors* of the articles and those who in other ways have contributed to this work. Especially, I would like to mention: *Anders Johansson*, *Håkan Askmark* and *Sten-Magnus Aquilonius* at the Unit of Neurology, UU, *Rolf Håkanson* at the Dept. of Pharmacology, LU, and past and present colleagues at the department(s): *Youri*, *Magnus P.*, *Jos*, *Jenny M.*, *Ardeshir*, *Stefan*, *My*, *Dan*, *Titti* and *Gabriel*.

I have also very much appreciated all invaluable computer support from *Mikael*, *Jonatan*, *Enrique* and *Ted*, and administrative assistance from *Barbro*.

Being a part of two groups involves many advantages, e.g., more colleagues to consult and two Christmas tables, but also a few drawbacks, i.e.,

cleaning-the-lab days and transportation between the departments during heavy rain and snowstorms. However, the advantages have always dominated! I would like to thank all *PhD-students, researchers, teachers and staff* at the *Division of Ionphysics* and at *Analytical Chemistry* for your friendship and collaboration.

Finally, I want to thank my safe and caring family; my parents *Elisabet* and *Kjell*, and my brothers *Erik* and *Lars*, for your enormous support. And my beloved *Mats*: This book is dedicated to you for all the joy and happiness you bring into my life. – What do you say when words are not enough?

9. Summary in Swedish

Analys av komplexa biologiska prover med vätskekromatografi och Fouriertransform-joncyklotronresonans-masspektrometri

Inledning

Proteiner är en grupp biologiska makromolekyler som deltar i de flesta livsviktiga processer i kroppen, såsom transport av näringsämnen och katalys av diverse kemiska reaktioner. För att bättre kunna förstå sambanden mellan till exempel olika sjukdomstillstånd och vår inre kemiska miljö är det av stor vikt att kunna identifiera och kvantifiera proteiner i kroppsvätskor och vävnader. Biologiska prover är oftast mycket komplexa och komponenterna uttrycks i varierande koncentrationer. Som exempel kan nämnas blodplasma, där det vanligast förekommande proteinet uttrycks i ca 10^{10} (10 000 000 000) gånger högre koncentration än proteinet interleukin. Dessutom utgör ett tiotal proteiner 90% av det totala proteininnehållet. Det krävs således mycket kraftfulla analysmetoder för att utföra proteomikstudier. Den här avhandlingen beskriver en speciell teknik för analys av proteiner och proteinmönster i komplexa biologiska prover, samt ger exempel på olika användningsområden.

Analysmetoden

Metoden som har använts bygger på vätskekromatografisk separation kombinerad med högupplösande masspektrometri. Vätskekromatografi (förkortas LC efter engelskans liquid chromatography) är en vanlig analytisk metod att separera komponenter i lösning från varandra. Experimentupställningen består huvudsakligen av en kolonn som innehåller en stationärfas samt en eller flera pumpar som levererar ett lösningsmedel, mobilfasen, vid högt tryck. Beroende på stationär- och mobilfasernas egenskaper fördelas mole-

kylerna olika starkt till dessa och kan separeras med avseende på hydrofobicitet.

En masspektrometer är en detektor som anger de analyserade molekylernas massa och laddning. Fouriertransform-joncyklotron-resonans-masspektrometri (FTICR MS) karaktäriseras av mycket hög upplösning, känslighet och massnoggrannhet. Massbestämningsfelet i ett välkalibrerat spektrum är runt 1 ppm (en tusendels promille). Den höga upplösningen möjliggör också att man kan skilja molekyler med liknande massor från varandra. Kombinationen LC-FTICR MS är mycket lovande för detektion av molekyler i komplexa prover.

Tillämpningar och metodförbättringar

Ett huvudtema i denna avhandling är analys av proteiner i kroppsvätskor, företrädesvis ryggmärgsvätska, men även blodplasma och fostervatten (**artiklar I-IV, VI**). I samtliga studier, har proteininnehållet först klyvts med ett enzym, trypsin, för att generera för masspektrometern mer hanterbara delar, tryptiska peptider. Trypsin klyver proteiner på förutsägbara, specifika positioner, och utgående från denna information kan teoretiska peptidmassor beräknas från kända proteinersekvenser.

Ungefär 6600 tryptiska peptider detekterades i ett ryggmärgsvätskeexperiment (**artikel I**), och närmare 40 proteiner kunde identifieras. Eftersom ryggmärgsvätskan står i kontakt med extracellulära ytan i hjärnan, kan proteinförändringar i vätskan korreleras med olika neurodegenerativa sjukdomar. I **artikel II** analyseras och jämförs proteininnehållet i ryggmärgsvätska från friska individer och patienter som lider av amyotrofisk lateralskleros (ALS). Ett mönsterigenkänningsprogram konstruerades för denna frågeställning, och karakteristiska skillnader kunde identifieras. Vid ett försök att klassificera ”okända” prover klassificerades 4 av 5 prover i rätt grupp. Dock kunde inte peptiderna som gav upphov till mönsterskillnaderna identifieras.

Vid kvantitativa analyser är det ofta önskvärt att kunna jämföra två prover under identiska experimentella betingelser. För att möjliggöra detta har metoder som bygger på kemisk inmärkning utvecklats. De två prover som ska jämföras får reagera med en tung respektive en lättare markör. Proverna blandas och analyseras därefter i samma experiment. På så sätt utesluter man variationer på grund av instrumentering och provmatris. I **artikel V** utvärderas en relativt ny markör för sådana ändamål. Metoden kunde med fördel kombineras med LC-FTICR MS, trots att eleringstiderna för tunga och lätta markörer skiljde sig med en knapp minut. Vidare studerades trypsinerings reproducerbarhet med denna och ytterligare en metod för proteinkvantifiering (**artikel VI**). En svårighet vid proteomik-studier är det breda dynamiska område som föreligger. I humana kroppsvätskor är serumalbumin det domi-

nerande proteinet. Ett flertal olika metoder har därför utvecklats för att avlägsna denna molekyl samt andra vanligt förekommande komponenter. Två olika tillvägagångssätts selektivitet och repeterbarhet utvärderas i **artikel IV**.

För att säkerställa peptiders identitet och karaktärisera delar av proteiner, är det vanligt att fragmentera dessa i masspektrometern. En metod för detta är fragmentering genom elektroninfångning (ECD). I avhandlingen beskrivs de första experimenten där LC-ECD-FTICR MS har sammankopplats för att generera sekvensinformation (**artikel VII**). Denna studie visar möjligheten att fragmentera standardpeptider och tryptiska peptider från enskilda proteiner. Den nyutvecklade metoden användes vidare för att studera naturligt förekommande peptider i bukspottskörtelvävnad från mus (**artikel VIII**). Metoden jämfördes med samma uppsättning kombinerad med en alternativ fragmenteringsmetod (nozzle-skimmer fragmentering). Dessa två tillvägagångssätt visade sig delvis komplettera varandra, även om effektiviteten var lägre i ECD-experimenten.

Slutsatser

Denna avhandling behandlar analys av komplexa biologiska prover med hjälp av vätskekromatografi kopplad till FTICR-masspektrometri. Målen med studierna har varit att identifiera och kvantifiera proteiner och peptider. Olika aspekter såsom provberedning, fragmentering och dataanalys har berörts. Med den experimentuppställning som presenteras i avhandlingen möjliggörs analys av små provvolymmer, analys tiden är relativt kort och provprepareringsstegen är snabba. Resultat från våra och andra gruppers experiment påvisar att kombinationen LC-FTICR MS har stor potential att bli betydande inom proteomikforskning. De begränsande faktorerna är idag främst datahantering samt att masspektrometern är relativt dyr och inte särskilt vanligt förekommande.

10. References

1. Kenyon GL, DeMarini DM, Fuchs E, Galas DJ, *et al.* *Mol. Cell Prot.* 2002; **1**: 763-780.
2. Anderson NL, Edwards JJ, Giometti CS, Willard KE, *et al.* *Electrophoresis '79* 1980; 313-328.
3. Clark BFC. *Nature* 1981; **292**: 491-492.
4. Anderson NG and Anderson L. *Clin. Chem.* 1982; **28**: 739-748.
5. Anderson NG, Matheson A and Anderson NL. *Proteomics* 2001; **1**: 3-12.
6. Graves PR and Haystead TAJ. *Microbiol. Mol. Biol. Rev.* 2002; **66**: 39-63.
7. McPherson JD, Marra M, Hillier L, Waterston RH, *et al.* *Nature* 2001; **409**: 934-941.
8. Venter C, Adams MD, Myers EW, Li PW, *et al.* *Science* 2001; **291**: 1304-1351.
9. Marko-Varga G and Fehniger TE. *J. Proteome Res.* 2004; **3**: 167-178.
10. de Hoffmann E and Stroobant V, *Mass Spectrometry*. 2002, Chichester, England: John Wiley & Sons.
11. Linschneid M. in *Handbook of Analytical Techniques* 2001, ed. H. Günzler and A. Williams, pp. 580-626.
12. Dole M, Mack LL, Hines RL, Mobley RC, *et al.* *J. Chem. Phys* 1968; **49**: 2240-2249.
13. Yamashita M and Fenn JB. *J. Phys. Chem.* 1984; **88**: 4451-4459.
14. Iribane JV and Thomson BA. *J. Chem. Phys* 1976; **64**: 2287-2294.
15. Thomson BA and Iribane JV. *J. Chem. Phys* 1979; **71**: 4451-4463.
16. Kebarle P and Pesche M. *Anal. Chim. Acta* 2000; **406**: 11-35.
17. He F, Hendrickson CL and Marshall AG. *Anal. Chem.* 2001; **73**: 647-650.
18. Belov ME, Gorshkov M, Udseth HR, Anderson GA, *et al.* *Anal. Chem.* 2000; **72**: 2271-2279.
19. Hipple JA, Sommer H and Thomas HA. *Phys. Rev.* 1949; **76**: 1877-1878.
20. Sommer H, Hipple JA and Thomas HA. *Phys. Rev.* 1951; **82**: 697-702.
21. Comisarow MB and Marshall AG. *Chem. Phys. Lett.* 1974; **25**: 282-283.
22. Amster IJ. *J. Mass Spectrom.* 1996; **31**: 1325-1337.
23. Marshall AG, Hendrickson CL and Jackson GS. in *Encyclopedia of Analytical Chemistry* 2000, ed. R. A. Meyers, pp. 11694-11728.
24. Horvath CG, Preiss BA and Lipsky SR. *Anal. Chem.* 1967; **39**: 1422-1428.
25. Vissers JPC, Claessens HA and Cramers CA. *J. Chromatogr. A* 1997; **779**: 1-28.
26. Chevret JP, Ursem M and Salzman JP. *Anal. Chem.* 1996; **68**: 1507-1512.
27. Stacey CC, Kruppa GH, Watson CH, Wronka J, *et al.* *Rapid Commun. Mass Spectrom.* 1994; **8**: 513-516.
28. Caravatti P and Allemann M. *Org. Mass Spectr.* 1991; **26**: 514-518.
29. Senko MW, Hendrickson CL, Emmett MR, Shi SD-H, *et al.* *J. Am. Soc. Mass Spectrom.* 1997; **8**: 970-976.

30. Emmett MR, White FM, Hendrickson CL, Shi SD-H, *et al.* *J. Am. Soc. Mass Spectrom.* 1998; **9**: 333-340.
31. Martin SE, Shabanowitz J, Hunt DF and Marto JA. *Anal. Chem.* 2000; **72**: 4266-4274.
32. Smith RD, Pasa-Tolic L, Lipton MS, Jensen PK, *et al.* *Electrophoresis* 2001; **22**: 1652-1668.
33. Shen Y, Tolic N, Zhao R, Pasa-Tolic L, *et al.* *Anal. Chem.* 2001; **73**: 3011-3021.
34. Shen Y, Zhao R, Belov ME, Conrads TP, *et al.* *Anal. Chem.* 2001; **73**: 1766-1775.
35. Palmblad M, Håkansson K, Håkansson P, Feng X, *et al.* *Eur. J. Mass Spectrom.* 2000; **6**: 267-275.
36. Nilsson S, Wetterhall M, Bergquist J, Nyholm L, *et al.* *Rapid Commun. Mass Spectrom.* 2001; **15**: 1997-2000.
37. Klose J. *Humangenetik* 1975; **26**: 231-243.
38. O'Farrell PH. *J. Biol. Chem.* 1975; **250**: 4007-4021.
39. Görg A, Weiss W and Dunn MJ. *Proteomics* 2004; **4**: 3665-3685.
40. MacCoss MJ, McDonald WH, Saraf A, Sadygov R, *et al.* *PNAS* 2002; **99**: 7900-7905.
41. Hancock WS, Wu S-L and Shieh P. *Proteomics* 2002; **2**: 352-359.
42. Wolters D, Washburn M and Yates JR. *Anal. Chem.* 2001; **73**: 5683-5690.
43. Smith RD, Anderson, G.A., Lipton, M.S., Pasa-Tolic, L., Shen, Y., Conrads, T.P., Veenstra, T.D., Udseth, H.R. *Proteomics* 2002; **2**: 513-523.
44. Bogdanov B and Smith RD. *Mass Spectrom. Rev.* 2005; **24**: 168-200.
45. Page JS, Masselon C and Smith RD. *Curr. Opin. Biotech.* 2004; **15**: 3-11.
46. Bergquist J. *Curr. Op. Mol. Ther.* 2003; **5**: 310-314.
47. Conrads TP, Anderson GA, Veenstra TD, Pasa-Tolic L, *et al.* *Anal. Chem.* 2000; **72**: 3349-3354.
48. Kelleher NL, Lin HY, Valaskovic GA, Aaserud DJ, *et al.* *J. Am. Chem. Soc.* 1999; **121**: 806-812.
49. Kelleher NL, Senko MW, Siegel MM and McLafferty FW. *J. Am. Soc. Mass Spectrom.* 1997; **8**: 380-383.
50. Tirumalai RS, Chan KC, Prieto DA, Issaq HJ, *et al.* *Mol. Cell Prot.* 2003; **2**: 1096-1103.
51. Anderson NL and Anderson NG. *Mol. Cell Prot.* 2002; **1**: 845-867.
52. Wikkelsö C. in *Neurologi* 1994, ed. S.-M. Aquilonius and J. Fagius, pp. 92-96.
53. Reiber H. *Clin. Chim. Acta* 2001; **310**: 173-186.
54. Thompson EJ, *The CSF proteins: A biochemical approach.* 1988, Amsterdam: Elsevier Science Publisher B.V..
55. Betz AL, Goldstein GW and Katzman R. in *Basic Neurochemistry: Molecular, Cellular and Medical Aspects, 5th ed.*, 1994, ed. G. J. Siegel, pp. 681-699.
56. Ostergard DR. *Obs. Gyn. Surv.* 1970; **25**: 297-319.
57. Gulbis B, Gervy C and Jauniaux E. *Early Hum. Dev.* 1998; **52**: 211-219.
58. Bonsnes RW. *Clin. Obstet. Gynecol.* 1966; **9**: 440-448.
59. Bergquist J, Palmblad M, Wetterhall M, Håkansson P, *et al.* *J. Mass Spectrom. Rev.* 2002; **21**: 2-15.
60. Palmblad M, Ramström M, Markides KE, Håkansson P, *et al.* *Anal. Chem.* 2002; **74**: 5826-5830.
61. Raymacker J, Daniels A, De Brabandere V and Missiaen C. *Electrophoresis* 2000; **21**: 2266-2283.
62. Sickmann A, Dormeyer W, Wortelkamp S, Woitalla D, *et al.* *Electrophoresis* 2000; **21**: 2721-2728.

63. Sickmann A, Dormeyer W, Wortelkamp S, Woitalla D, *et al.* *J. Chromatogr. B* 2002; **771**: 167-196.
64. Yuan X, Russell T, Wood G and Desiderio D. *Electrophoresis* 2002; **23**: 1185-1196.
65. Wetterhall M, Palmblad M, Håkansson P, Markides KE, *et al.* *J. Proteome Res.* 2002; **1**: 361-366.
66. Terry DE and Desiderio DM. *Proteomics* 2003; **3**: 1962-1979.
67. Yuan X and Desiderio DM. *J. Chromatogr. B* 2005; **815**: 179-189.
68. Davidsson P, Folkesson S, Christiansson M, Lindbjer M, *et al.* *Rapid Commun. Mass Spectrom.* 2002; **16**: 2083-2088.
69. Folkesson Hansson S, Puchades M, Blennow K, Sjögren M, *et al.* *Proteome Science* 2004; **2**: 1-11.
70. Liberatori S, Bini L, de Felice C, Magi B, *et al.* *Electrophoresis* 1997; **18**: 2816-2822.
71. Stewart CJ, Iles RK and Perrett D. *Electrophoresis* 2001; **22**: 1136-1142.
72. Travis J, Bowen J, Tewksbury D, Johnson D, *et al.* *Biochem. J.* 1976; **157**: 301-306.
73. Steel LF, Trotter MG, Nakajima PB, Mattu TS, *et al.* *Mol. Cell Prot.* 2003; **2**: 262-270.
74. Greenough C, Jenkins RE, Kitteringham NR, Pirmohamed M, *et al.* *Proteomics* 2004; **4**: 3107-3111.
75. Wang YY, Cheng P and Chan DW. *Proteomics* 2003; **3**: 243-248.
76. Chromy BA, Gonzales AD, Perkins J, Choi MW, *et al.* *J. Proteome Res.* 2004; **3**: 1120-1127.
77. Sobek O and Adam P. *J. Neurol.* 2003; **250**: 371-372.
78. Kay AD, Petzold A, Kerr M, Keir G, *et al.* *J. Neurotrauma* 2003; 243-250.
79. Terrisse L, Poirier J, Bertrand P, Merched A, *et al.* *J. Neurochem.* 1998; **71**: 1643-1650.
80. Smyth MD, Cribbs DH, Tenner AJ, Shankle WR, *et al.* *Neurobiol. Aging* 1994; **15**: 609-614.
81. Tenhunen R, Iivanainen M and Kovanen J. *Acta Neurol Scand.* 1978; **58**: 366-373.
82. Petricoin EE, Paweletz CP and Liotta LA. *J. Mammary Gland Biol. Neoplasia* 2002; **7**: 433-439.
83. Brisby H, Olmarker K, Rosengren L and Cederlund C-G. *Spine* 1999; **24**: 742-746.
84. Rosengren LE, Karlsson J-E, Karlsson J-O, Persson LI, *et al.* *J. Neurochem.* 1996; **67**: 2013-2018.
85. Hunter TC, Andon NL, Koller A, Yates JR, *et al.* *J. Chromatogr. B* 2002; **782**: 165-181.
86. Bischoff R and Luidert TM. *J. Chromatogr. B* 2004; **803**: 27-40.
87. Radulovic D, Jelveh S, Ryu S, G. HT, *et al.* *Mol. Cell Prot.* 2004; **3**: 984-997.
88. Wang W, Zhou H, Lin H, Roy S, *et al.* *Anal. Chem.* 2003; **75**: 4818-4826.
89. Ekegren T, *Transmethylation, polyamines and apoptosis in amyotrophic lateral sclerosis.* Acta Universitatis Upsaliensis. 2004, Uppsala.
90. Hand CK, Khoris J, Salachas F, Gros-Louis F, *et al.* *Am. J. Hum. Genet.* 2002; **70**: 251-256.
91. Rosen DR, Siddique T, Patterson D, Figlewicz DA, *et al.* *Nature* 362; **362**: 59-62.
92. Rachakonda V, Pan TH and Le WD. *Cell Research* 2004; **14**: 349-360.
93. Lewy F. in *Handbuch der Neurologie III* 1912, ed. pp. 920-933.

94. Lovestone S and McLoughlin DM. *J Neurol Neurosurg Psychiatry* 2002; **72**: 152-161.
95. Al-Chalabi A and Miller CCJ. *BioEssays* 2003; **24**: 346-355.
96. Brooks B. *J Neurol Sci* 1994; **124**: Suppl: 96-107.
97. Regnier FE, Riggs L, Zhang R, Xiong L, *et al.* *J. Mass Spectrom.* 2002; **37**: 133-145.
98. Gygi SP, Rist B, Gerber SA, Turecek F, *et al.* *Nat. Biotechnol.* 1999; **17**: 994-999.
99. Cagney G and Emili A. *Nat. Biotechnol.* 2002; **20**: 163-170.
100. Conrads TP, Alving K, Veenstra TD, Belov ME, *et al.* *Anal. Chem.* 2001; **73**: 2132-2139.
101. Freeman WM and Hemby SE. *Neurochem. Res.* 2004; **29**: 1065-1081.
102. Ünlü M, Morgan ME and Minden JS. *Electrophoresis* 1997; **18**: 2071-2077.
103. Zhou H, Ranish JA, Watts JD and Aebersold R. *Nat. Biotechnol.* 2002; **19**: 512-515.
104. Hansen KC, Schmitt-Ulms G, Chalkley RJ, Hirsch J, *et al.* *Mol. Cell Prot.* 2003; **2**: 299-314.
105. Yao X, Freas A, Ramirez J, Demirev PA, *et al.* *Anal. Chem.* 2001; **73**: 2836-2842.
106. Mirgorodskaya OA, Kozmin YP, Titov MI, Körner R, *et al.* *Rapid Commun. Mass Spectrom.* 2000; **14**: 1226-1232.
107. Ji J, Chakraborty A, Geng M, Zhang X, *et al.* *J. Chromatogr. B* 2000; **745**: 197-210.
108. Münchbach M, Quadroni M, Miotto G and James P. *Anal. Chem.* 2000; **72**: 4047-4057.
109. Beardsley RL and Reilly JP. *J. Proteome Res.* 2003; **2**: 15-21.
110. Thumm M, Hoenes J and Pfeleiderer G. *Biochim. Biophys. Acta* 1987; **923**: 263-267.
111. Matsuda K, Yanagisawa I, Isomura Y, Mase T, *et al.* *Synth. Commun.* 1997; **27**: 2393-2402.
112. Sleno L and Volmer DA. *J. Mass Spectrom.* 2004; **39**: 1091-1112.
113. Roepstorff P and Fohlman J. *Biomed. Mass Spectrom.* 1984; **11**: 601.
114. Little DP, Speir JP, Senko MW, O'Connor PB, *et al.* *Anal. Chem.* 1994; **66**: 2809-2815.
115. Li W, Hendrickson CL, Emmett MR and Marshall AG. *Anal. Chem.* 1999; **71**: 4397-4402.
116. Price WD, Schnier PD and Williams ER. *Anal. Chem.* 1996; **68**: 859-866.
117. Sannes-Lowery K, Griffey RH, Kruppa GH, Speir JP, *et al.* *Rapid Commun. Mass Spectrom.* 1998; **12**: 1957-1961.
118. Li L, Masselon C, Anderson GA, Pasa-Tolic L, *et al.* *Anal. Chem.* 2001; **73**: 3312-3322.
119. Tanaka Y, Sato I, Iwai C, Kosaka T, *et al.* *Anal. Biochem.* 2001; **293**: 157-168.
120. Zubarev RA, Kelleher NL and McLafferty FW. *J. Am. Chem. Soc* 1998; **120**: 3265-3266.
121. Zubarev RA. *Curr. Op. Biotech.* 2004; **15**: 12-16.
122. Baba T, Hashimoto Y, Hasegawa H, Hirabayashi A, *et al.* *Anal. Chem.* 2004; **76**: 4263-4266.
123. Silivra OA, Kjeldsen F, Ivonin IA and Zubarev RA. *J. Am. Chem. Soc* 2005; **16**: 22-27.
124. Zubarev RA. in *Mass Spectrometry and Hyphenated Techniques in Neuropeptide Research* 2002, ed. pp. 277-295.

125. Tsybin YO, Håkansson P, Budnik BA, Haselmann KF, *et al.* *Rapid Commun. Mass Spectrom.* 2001; **15**: 1849-1854..
126. Davidson W and Frego L. *Rapid Commun. Mass Spectrom.* 2002; **16**: 993-998.
127. Tsybin YO, Håkansson P, Wetterhall M, Markides KE, *et al.* *Eur. J. Mass Spectrom.* 2002; **8**: 389-395.
128. Perkins DN, Pappin DJ, Creasy DM and Cottrell JS. *Electrophoresis* 1999; **20**: 3551-3567.
129. Banting FG and C.H. B. *J. Lab. Clin. Med.* 1922; **7**: 251-266.
130. Rosenfeld L. *Clin. Chem.* 2002; **48**: 2270-2288.
131. Clynen E, De Loof A and Schoofs L. *Gen. Comp. Endocrinol.* 2003; **132**: 1-9.
132. Schrader M and Schultz-Knappe P. *Trends Biotech.* 2001; **19**: S55-60.
133. Zubarev RA, Kruger NA, Fridriksson EK, Lewis MA, *et al.* *J. Am. Chem. Soc* 1999; **121**: 2857-2862.
134. Guan Z, Yates NA and Bakhtiar R. *J. Am. Soc. Mass Spectrom.* 2003; **14**: 605-613.
135. Stephenson JL, Cargile BJ and McLuckey SA. *Rapid Commun. Mass Spectrom.* 1999; **13**: 2040-2048.
136. Verchere CB, Paoletta M, Neerman-Arbez M, Rose K, *et al.* *J Biol Chem.* 1996; **271**: 27475-27481.
137. Paoletta M, Kahn SE and Halban PA. *Diabetologia* 2002; **45**: 1523-1527.
138. Pedrioli PGA, Eng JK, Hubley R, Vogelzang M, *et al.* *Nature Biotech.* 2004; **22**: 1459-1466.

Acta Universitatis Upsaliensis

*Digital Comprehensive Summaries of Uppsala Dissertations
from the Faculty of Science and Technology 31*

Editor: The Dean of the Faculty of Science and Technology

A doctoral dissertation from the Faculty of Science and Technology, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology. (Prior to January, 2005, the series was published under the title "Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology".)

Distribution: publications.uu.se
urn:nbn:se:uu:diva-5729



ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2005